VOL. XXVII NOVEMBER, 1916 No. 2

NOV 16 1916
UNIV. OF MICH.
BIOLOGICAL

THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

EDITED BY

H. D. DAKIN, New York City.
E. K. DUNHAM, New York City.
LAFAYETTE B. MENDEL, New Haven, Conn.
A. N. RICHARDS, Philadelphia, Pa.
DONALD D. VAN SLYKE, New York City.
CLARENCE J. WEST, New York City.

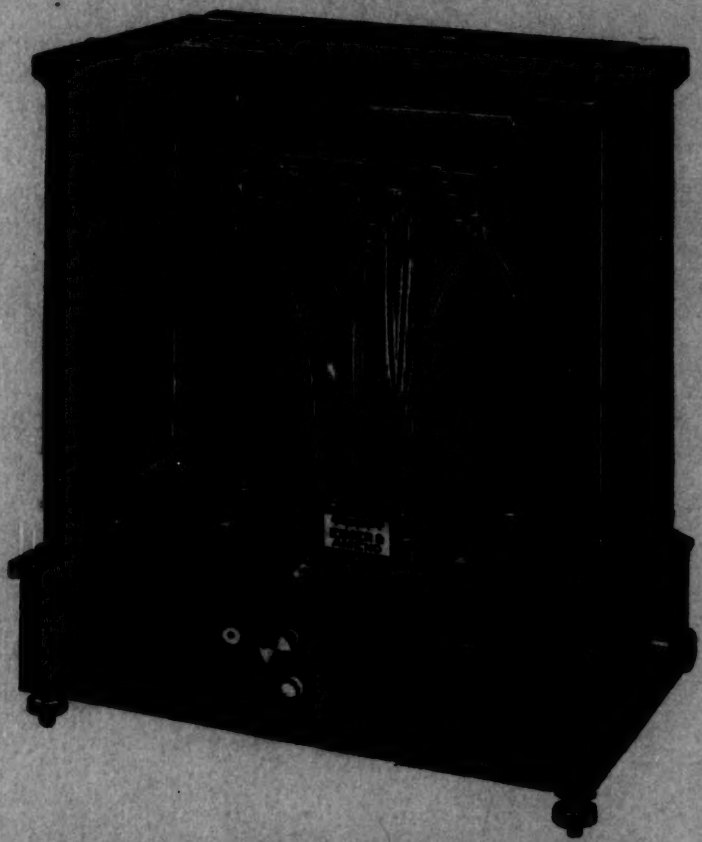
WITH THE COLLABORATION OF

J. J. ABEL, Baltimore, Md.
R. H. CHITTENDEN, New Haven, Conn.
OTTO FOLIN, Boston, Mass.
WILLIAM J. GIES, New York.
L. J. HENDERSON, Cambridge, Mass.
REID HUNT, Boston, Mass.
W. A. JACOBS, New York.
WALTER JONES, Baltimore, Md.
J. B. LEATHES, Sheffield, England.
P. A. LEVENE, New York.
JACQUES LOEB, New York.
A. S. LOEVENHART, Madison, Wis.
GRAHAM LUSK, New York.
A. B. MACALLUM, Toronto, Canada.
J. J. R. MACLEOD, Cleveland, Ohio.
JOHN A. MANDEL, New York.
A. P. MATHEWS, Chicago, Ill.
F. G. NOVY, Ann Arbor, Mich.
THOMAS B. OSBORNE, New Haven, Conn.
T. BRAILSFORD ROBERTSON, Berkeley, Cal.
P. A. SHAFFER, St. Louis, Mo.
A. E. TAYLOR, Philadelphia, Pa.
F. P. UNDERHILL, New Haven, Conn.
V. C. VAUGHAN, Ann Arbor, Mich.
ALFRED J. WAKEMAN, New Haven, Conn.

PUBLISHED MONTHLY
BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
FOR THE JOURNAL OF BIOLOGICAL CHEMISTRY, INC.
AT 2419-21 GREENMOUNT AVENUE, BALTIMORE, MD.

Copyright 1916 by The Journal of Biological Chemistry

Entered as second-class matter, August 1, 1911, at the Post Office at Baltimore, Md., under the Act of March 3, 1879.



CHAINOMATIC
ANALYTICAL BALANCE

The marked advantages of this Chain-Vernier system have quickly been realized by Educational and Research Laboratory workers, evidenced by the numerous orders and repeat orders received. Elimination of the customary troublesome riders and small fraction weights up to 50 mgr. allows more accurate weighings to be made and with remarkable rapidity which adds to the accuracy.

E. & A. No. 221. Capacity 200 grams. Sensitiveness $\frac{1}{10}$th mgr. Diameter of Pans 2$\frac{3}{4}$ inches. Length of Beam 7 inches Agate planes and knife edges. Capacity of bar and vernier 50 mgr. to $\frac{1}{10}$th mgr. Polished mahogany case, front sliding frame, counterpoised. Mounted on black glass plate. **$165.00**

E. & A. No. 221/1. Ditto, gold plated beam, pillar, pans, etc..... **165.00**

E. & A. No. 213/2. Capacity 100 grms. Sensitiveness $\frac{1}{10}$th mgr. Length of Beam 6$\frac{1}{2}$ inches, otherwise similar to No. 221..... **120.00**

Descriptive Pamphlet on Request

EIMER & AMEND

FOUNDED 1851

Laboratory Supplies

NEW YORK and PITTSBURGH, PA.

THE RELATION OF OXIDASE REACTIONS TO CHANGES IN HYDROGEN ION CONCENTRATION.

By GUILFORD B. REED.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

(Received for publication, September 11, 1916.)

Biological oxidations are known to be markedly affected by acids and alkalies. Loeb and Wasteneys[1] have shown that when sufficient HCl to give a hydrogen ion concentration of 1×10^{-4} M was added to unfertilized *Arbacia* eggs contained in a balanced salt solution only about half as much oxygen was absorbed as was absorbed by the same eggs when in neutral solution. On the other hand, when sufficient NaOH was added to give a hydroxyl ion concentration of 4.2×10^{-4} M the rate of oxygen absorption was approximately doubled; and when the hydroxyl ion concentration was increased to 8×10^{-4} two and three-fourths times more oxygen was absorbed than by the same eggs when in neutral solution. Similar results have been obtained in other cases.

It is a natural assumption that alkali directly influences the rate of oxidation. Several investigators have indicated that the oxidases are affected by changes in the acidity of the solution in which they act. Bertrand[2] found that laccase from *Rhus succedanea* acting on guaiacol was completely inhibited when sufficient H_2SO_4 was added to make the concentration 0.002 M. Abderhalden and Guggenheim[3] found that tyrosinase from various sources was destroyed by 0.01 M HCl and its action considerably retarded by 0.01 M NaOH. Wolff[4] has also pointed out that tyrosinase from *Russula delica* is most active when the solution is neutral to phenolphthalein.

[1] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxvii, 410.

[2] Bertrand, G., and Muttermilch, *Compt. rend. Acad.*, 1907, cxliv, 1385.

[3] Abderhalden, E., and Guggenheim, M., *Z. physiol. Chem.*, 1907–08, liv, 331.

[4] Wolff, J., *Compt. rend. Acad.*, 1909, cxlviii, 500.

In the experiments just mentioned the acidity or alkalinity was determined by the amount of standardized acid or alkali added to definite amounts of the solution containing the ferment, on the assumption that the dilution of the standardized acid by the mixture had the same effect as an equal dilution with distilled water.

The writer finds that this assumption is incorrect, as is clearly shown by determinations with a hydrogen electrode. The following example illustrates this.

An extract of potato (prepared by mixing equal volumes of grated potato and distilled water, allowing the mixture to stand for an hour, and filtering) was added to an equal volume of 0.01 M HCl. Since the acid was diluted to twice the original volume it would then be 0.005 M ($C_H = 5 \times 10^{-3}$) if the potato extract acted like distilled water. The hydrogen electrode measurement, however, showed the concentration to be only 5×10^{-4}. The presence of the potato has therefore reduced the acidity to one-tenth, although the extract itself is very nearly neutral. This effect is due to the protein present in the solution as well as the presence of other amphoteric electrolytes, including probably phosphates and carbonates.

In view of this observation it seemed desirable to repeat some of the earlier experiments to determine the real relation of the oxidases to acidity. A potato extract, prepared by grating the tuber with an equal volume of water and quickly filtering, was divided into several portions of 50 cc. each. These were placed in beakers, and sufficient HCl or NaOH was added to give the hydrogen ion concentration (as measured by the gas chain) shown in the first column of Table I. These were then exposed at room temperature and the rate of oxidation of the naturally occurring chromogen was followed by observing the appearance of red and finally black colors. It will be seen from the table that a hydrogen ion concentration of 5.5×10^{-4} was sufficient to inhibit the reaction completely.

Immediately after adding the acid to these extracts portions of 10 cc. were removed and 1 cc. of 2 per cent gum guaiac tincture was added to each. The results stated in the last column of Table I indicate that the same concentrations of acid which inhibited its reaction with the chromogen inhibit also the oxidation of gum guaiac.

TABLE I.

H ion concentration.	Oxidation of chromogen.		Oxidation of gum guaiac, 5 min.
	12 hrs.	24 hrs.	
1.5×10^{-3}	No color.	No color.	No color.
7.6×10^{-4}	" "	" "	" "
5.5×10^{-4}	" "	" "	" "
1.5×10^{-4}	Faint.	Red.	Blue.
7.5×10^{-5}	Red.	Black.	Deep blue.
2×10^{-7}	Deep red.	"	" "
1×10^{-10}	Red.	"	" "

To the three preparations, the oxidase action of which had been inhibited by contact with the acid for 12 hours, sufficient NaOH was added to neutralize to phenolphthalein. Portions of 10 cc. were then removed and mixed with gum guaiac, and the balance was exposed for another 12 hours. From the results stated in Table II it appears that the highest concentrations of acid destroyed the ferments, since neutralizing failed to revive them; the concentrations just sufficient to inhibit the oxidations, however, did not affect them irreversibly.

TABLE II.

Exposed for 12 hrs. H ion concentration.	Oxidation after neutralization.	
	Chromogen.	Gum guaiac.
1.5×10^{-3}	No color.	No color.
7.6×10^{-4}	Red.	Blue.
5.5×10^{-4}	"	"

Table III shows the results of an experiment with an extract of ripe Astrachan apples (prepared by grating the fruit with an equal volume of water and filtering). The extract was divided into several portions of 50 cc. each and sufficient acid or alkali was added to give the concentrations stated in the table (measured by the gas chain).

These results show that the concentrations of acid which are needed to inhibit oxidases are much lower than those stated by previous investigators; this is doubtless due to their faulty methods of measurement. Bertrand stated 0.002 M to be the lowest

TABLE III.

H ion concentration.	Oxidation.		
	Chromogen.		Gum guaiac.
	12 hrs.	48 hrs.	
1.6×10^{-3}	No color.	No color.	No color.
7.4×10^{-4}	" "	" "	" "
3.8×10^{-4}	Faint.	Faint.	Blue.
8×10^{-5}	Red.	Red.	Deep blue.
1.5×10^{-5}	"	Red-brown.	" "
1×10^{-7}	"	"	" "
2×10^{-9}	"	"	" "

concentration which completely inhibited oxidases and Colin and Sénéchal[5] report that horse-radish peroxidase had very little action in 0.004 M acid; while the present experiment indicates that 0.0005 to 0.007 M was sufficient to prevent the action of certain oxidases. It is therefore evident that the optimal activity of these oxidases is reached when they are in a medium which is very nearly neutral or slightly alkaline.

On this basis it may be possible to explain the remarkable activation of oxidation processes by the addition of exceedingly small amounts of alkali, as in the experiments of Loeb and Wasteneys on Echinoderm eggs. It is conceivable that in the unfertilized eggs the hydrogen ion concentration is too high for the rapid operation of oxidases, but that the added alkali changes this to the optimum condition.

All the ferments which have been investigated in this respect exhibit their maximum activity at a definite hydrogen ion concentration, and it was to be expected that the oxidases would behave similarly. Hudson[6] regards the activity of invertase (which behaves as an amphoteric electrolyte and hence is capable of combining with acids and bases) as proportional to the amount of ferment not so combined. This may apply to other ferments but it has not yet been shown to do so.

[5] Colin, H., and Sénéchal, A., *Compt. rend. Acad.*, 1911, cliii, 76; *Rev. gén. Bot.*, 1912, xxiv, 49.

[6] Hudson, C. S., *J. Am. Chem. Soc.*, 1910, xxxii, 1220.

PHOSPHATIDES IN THE DUCTLESS GLANDS.

By FREDERIC FENGER.

(From the Research Laboratory in Organotherapeutics of Armour and Company, Chicago.)

(Received for publication, September 5, 1916.)

This investigation was carried out to determine the amounts of phosphatides present in the ductless glands and the relation of the results to the functioning of these glands. Inasmuch as the work was of a preliminary nature no attempts were made to separate or isolate the groups of phosphatides from other extractive matters such as fat. Petroleum ether was chosen as a general solvent. Ordinary muscle fiber in the form of straight lean meat (chucks) was used as a standard, and brain tissue and spinal cord were employed for comparative purposes as examples of tissues exceptionally rich in phosphatides. Cattle glands are especially adapted for this work on account of their size and comparative freedom from adipose tissue. The following glands were used: Pineal gland from cattle; pituitary body, anterior and posterior lobes from cattle, and for comparative purposes also the anterior lobe from young calves; thyroid from cattle; thymus from young growing calves; suprarenals from cattle; and the true corpus luteum from pregnant cows. The thymus was selected from young calves 2 to 4 months old, the early growth period during which this gland is most active.

The various glands, from several hundred animals, were collected shortly after slaughtering and while still retaining the animal heat, and were carefully trimmed. In the case of the pituitary body, the outside capsule was discarded and the anterior and posterior lobes represent glandular tissue only. The corpora lutea were obtained by splitting the ovaries and removing the soft orange-colored glandular portion of the corpus luteum. The suprarenals also were skinned so that the portion employed represents the medulla and cortex only. The pineal and the

thyroid were both trimmed as free as possible from all adherent connective and fatty tissues. The entire brain was employed, the outside tissue removed, and the brain washed with water until free from blood. The spinal cord represents the entire spinal marrow.

The various lots of glands and tissues were finely minced and desiccated *in vacuo* at 35–37°C. to constant weight, then coarsely ground and extracted with petroleum ether in a Soxhlet apparatus. After extraction, the various lots of petroleum ether containing the soluble portions of the glands were filtered and the solvent evaporated, first by a current of air and slight heating on a water bath and then by further drying *in vacuo* to constant weight. On these residues the total P_2O_5 was determined and the equivalent amounts of lecithin were obtained by multiplying the P_2O_5 figures by 11.24. These lecithin figures do not necessarily represent the actual amounts of lipoids present; they merely serve as a general guide for the present investigation. The phosphorus was determined on 1 gm. samples by boiling with 2 cc. of concentrated sulfuric acid and several small portions of nitric acid until oxidation was complete. The digested liquids were then diluted with water and the phosphorus was determined according to the official volumetric method.

In the tabulation will be found the loss of moisture and the percentage of petroleum ether-soluble substances in the fresh glands together with the yield of desiccated fat-free material, also the phosphorus in the extracted residue and the equivalent percentages of lecithin, both calculated on the basis of the residue and the fresh tissue.

The most striking feature of the tabulated figures is the fact that thyroid tissue contains no more phosphatides than ordinary muscle fiber, whereas all the other ductless glands show much larger amounts. The desiccated thyroid contained 0.41 per cent of iodine, indicating that the gland was exceedingly active physiologically and contained a maximum of active principles. This seems to be convincing evidence that the lipoids play no important part in the internal chemistry of the thyroid.

The petroleum ether-soluble portion of the muscle fiber as well as the thyroid consisted principally of fat. The color was light yellowish brown and the consistency that of ordinary beef fat.

	Fresh tissue.				Petroleum ether-extracted material.				
	Moisture.	Petroleum ether-soluble.	Desiccated fat-free material.	Phosphatides calculated as lecithin in fresh tissue.	P_2O_5	Lecithin ($P_2O_5 \times 11.24$).	Consistency.	Color.	Odor.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*			
Pituitary body, anterior lobe, calves.....	81.00	2.30	16.70	1.44	5.57	62.61	Brain lecithin.	Yellowish brown.	Brain lecithin.
Pituitary body, anterior lobe, cattle	76.20	2.90	20.90	1.65	5.10	57.32	" "	" "	" "
Pituitary body, posterior lobe, cattle*...	82.60	1.80	15.60	0.96	4.74	53.28	" "	" "	" "
Pineal body, cattle.....	81.60	2.80	15.60	1.79	5.70	64.10	" "	" "	" "
Thyroid, cattle†.....	75.50	4.20	20.30	0.13	0.27	3.03	Semisolid, principally fat.	Light yellowish brown.	Beef fat, slight odor of lecithin.
Thymus, calves.....	81.30	1.67	17.03	0.43	2.31	25.96	Semisolid, greasy.	Brownish yellow.	Lecithin.
Suprarenals, cattle‡ ...	75.40	4.43	20.17	2.37	4.75	53.39	Pasty.	Brownish red.	Crude lecithin.
Corpus luteum, pregnant cows.....	78.00	4.84	17.16	2.12	3.90	43.84	Semiliquid, pasty.	Reddish brown.	" "
Muscle fiber, lean meat.	74.20	1.44	24.36	0.14	0.85	9.55	Semisolid, principally fat.	Yellowish brown.	Beef fat, slight odor of lecithin.
Brain, cattle.....	76.90	10.78	12.32	5.70	4.70	52.83	Crude brain lecithin.	Light yellowish brown.	Brain lecithin.
Spinal cord, cattle.....	65.35	27.60	7.05	14.17	4.57	51.36	Crude brain lecithin.	Light yellowish brown.	" "

* Normal uterine-contracting power.

† 0.41 per cent iodine in desiccated glands.

‡ 0.9 per cent epinephrin in desiccated glands.

The infant thymus contained about three times as much phosphatides as lean meat from adult animals of the same species. The thymus fat was brownish yellow and possessed an odor resembling lecithin.

The anterior lobe of the pituitary body contained approximately ten times and the posterior lobe seven times as much phosphatides as straight lean meat. The posterior lobe showed the usual amount of uterine-contracting active principle, indicating normal physiological activity of the gland. The isolated uterus method as outlined by Roth[1] was employed in this work. The petroleum ether-soluble portion of this gland, both anterior and posterior lobes, resembled brain lecithin in consistency. The color was light yellowish brown and the odor similar to lecithin.

The pineal body showed about thirteen times more phosphatides than did muscle tissue. The petroleum ether-soluble portion resembled crude lecithin from brain tissue both in consistency, color, odor, and taste.

The corpus luteum of pregnancy was also rich in phosphatides, containing fifteen times as much as muscle tissue. The petroleum ether-soluble portion was of semisolid pasty consistency and reddish brown color, and possessed a strong lecithin odor.

The suprarenals were the richest in phosphatides of all the ductless glands, containing seventeen times more than ordinary muscle tissue. The petroleum ether-soluble portion was of pasty or waxy consistency, brownish red color, and pronounced lecithin odor.

The epinephrin content was determined by the manganese dioxide method described by Seidell.[2] The desiccated gland contained 0.9 per cent epinephrin, which means full physiological activity.

There was very little difference in the phosphorus contents of the extracted portions from brain and spinal cord, but the spinal cord contained almost three times as much petroleum ether-soluble substance as the brain. The soluble portions of the brain and spinal cord were both of waxy consistency, resembling

[1] Roth, G. B., *Bull. Hyg. Lab.*, No. 100, 1914.

[2] Seidell, A., *J. Biol. Chem.*, 1913, xv, 197.

soft paraffin, of light yellowish brown color, and characteristic lecithin odor.

There are no conclusive evidences that the pineal body and the thymus contain active principles of physiological significance. The corpus luteum has given some proof of physiological importance, but no active principle or means of standardizing the product for medicinal purposes have yet been discovered or outlined. In the pituitary body, the thyroid, and the suprarenals, however, we have definite means of judging the therapeutic activity of their internal secretions and it is from these glands that the present conclusions must be drawn. The analytical data show that these glands contain high amounts of active principles and consequently possess full physiological activity. The pituitary body and the suprarenals as well as the pineal, the thymus, and the corpus luteum of pregnancy contain considerable amounts of phosphatides in excess of ordinary muscle tissue and it is safe to assume, therefore, that the phosphatides play some part in the internal chemistry of all the ductless glands, with the exception of the thyroid.

SUMMARY.

The pituitary body and the suprarenals, as well as the pineal, the infant thymus gland, and the corpus luteum of pregnancy contain considerable amounts of phosphatides in excess of ordinary muscle tissue, indicating that the phosphatides play some part in the internal chemistry of these ductless glands. The thyroid does not contain any more phosphatides than straight lean meat and it may, therefore, be assumed that this gland performs its secretory functions independent of the phosphatides.

NUTRITION AND EVOLUTION.

SECOND NOTE.

By JACQUES LOEB and J. H. NORTHROP.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 9, 1916.)

In a former paper Loeb[1] raised the question whether or not the evolution of animals as high in the scale of life as insects was possible without the existence of green plants. It is generally assumed in speculations on evolution that chlorophyll preceded the evolution of animals. Attacked from this point the question of evolution becomes a question of nutrition, or rather of the synthesis of the highly complex proteins of the body.

Loeb had found that sterile culture media like those used by Pasteur for the raising of yeast were also sufficient for the raising of flies—*Drosophila* was used for this purpose. It was pointed out that this did not prove that the power of synthesis of the flies is the same as that of the yeast since although the culture media were sterile, the flies were not; and that it was possible that the flies carried microorganisms which synthesized the proteins for them.[1] The question could only be decided by experiments with flies absolutely free from parasitic or symbiotic microorganisms.

Bogdanow[2] was the first to attempt to raise flies free from bacteria. He experimented on the blowfly, *Calliphora vomitoria*. His method consisted in sterilizing the eggs with solutions of $HgCl_2$. He found that sterile larvæ of the fly grew only very slowly on sterile meat, never reaching their full size. They died without undergoing metamorphosis. He assumes that they require the aid of bacteria for their normal nutrition. Wollman[3] repeated the experiments of Bogdanow, confirming his

[1] Loeb, J., *J. Biol. Chem.*, 1915, xxiii, 431.

[2] Bogdanow, *Arch. Physiol., Suppl.*, 1908, 173.

[3] Wollman, E., *Ann. l'Inst. Pasteur*, 1911, xxv, 79.

results. Delcourt and Guyénot[4] experimented with *Drosophila*. These authors first tried Bogdanow's method but did not succeed since the antiseptic treatment killed all the eggs of *Drosophila*. They proceeded by frequent transference of flies raised on acid media and thus finally obtained some colonies which on microscopic and bacteriological test proved sterile. Guyénot observed that sterile flies grew on yeast, living or dead, and that after several generations they also grew well on sterile potatoes. The larvæ grew well on peptone and salts but did not metamorphose.

We returned to the method of Bogdanow which seemed more direct and reliable, by raising sterile cultures from eggs sterilized in $HgCl_2$. Sterilized banana was covered with wet filter paper and female flies were put in the flasks with the banana. After several hours the eggs were taken from the filter paper and placed in 0.1 per cent $HgCl_2$ or a saturated solution of $HgCl_2$ in alcohol for 6 to 7 minutes. Most eggs are killed by the treatment but a small percentage recover and develop when put into a flask containing sterilized yeast and cotton. They grew and metamorphosed entirely normally on the following culture media:

Sterilized bakers' yeast	450 gm.
Water	1,000 cc.
Citric acid	50 gm.

The acid was added solely for the purpose of diminishing the chance of the development of bacteria when the flies were removed from the flask. The presence of citric acid diminished the number of larvæ (and probably also the number of eggs laid), since in the cultures without citric acid the number of larvæ and flies produced was greater. But cultures without citric acid usually became infected with bacteria when the old flies were removed from the flask for the purpose of starting new cultures. We have thus far raised twelve successive generations free from all microorganisms.

Our tests for the sterility of the flies consisted in putting the dead flies as well as parts of the culture into glucose agar, agar, plain broth, litmus milk, glucose broth, and potato tubes. All

[4] Delcourt, A., and Guyénot, E., *Bull. Sc. France et Belgique*, 1911, xlv, 249. Guyénot, E., *Compt. rend. Soc. biol.*, 1913, lxv, pt. i, 97, 178, 223, 270; 1914, lxvi, pt. i, 483, 548.

these tests remained permanently sterile. In addition, smears were made from the flies, pupæ, and larvæ, and examined. They were free from bacteria and fungi.

We made a large number of culture experiments with filter paper, cane sugar, $MgSO_4$, KH_2PO_4, NaCl, $CaCl_2$, and nitrogenous compounds as indicated in the following table. The sterile flies were transferred to the tubes containing the culture media with sterile pipettes, following the procedure of Guyénot in the manipulation of the flies.

Substrate.	Result.
Casein	Some larvæ reach full size but do not metamorphose.
Edestin	"
Egg albumin	"
Mixture of leucine, alanine, glycine, asparagine, tyrosine, tryptophane, and histidine[5]	"
Milk	"

Hence in none of these media was it possible to raise a single fly or pupa.

We then made experiments with sterile bananas and sterile potatoes. Bananas are the usual culture medium used for raising non-sterile *Drosophila* in large numbers. The results of our experiments on sterile bananas with sterilized flies were most remarkable. The larvæ grew slowly and very few pupated. Some larvæ remained alive 20 to 30 days. The flies which hatched from the pupæ were very small, about one-fourth the normal size, and had very little pigment. It was impossible to raise a second generation from them.

They were crossed with normal flies and cultivated on sterile yeast, but although both crosses were tried, no larvæ were obtained. When the females were normal flies and the males flies raised on sterile bananas, a few eggs were laid, but no larvæ hatched. This indicates that flies, free from microorganisms (yeast), when raised on sterile bananas or sterile potatoes were also sexually sterile. It is of course possible that this negative

[5] Some of these pure amino-acids were kindly given us by Dr. P. A. Levene.

result is not final although our conclusion is based on the result of ten different cultures.

Yeast was then the only medium on which normal larvæ could be raised with these sterile cultures of flies and it seems to be the indispensable food for these insects. Yeast must therefore contain some substance required for their growth and this substance must be rather resistant to heat since yeast heated for 1 hour at 120° is an excellent culture medium. We tried to isolate this substance from the yeast. Yeast extracted with boiling alcohol for 48 hours or with cold alcohol for 10 days was no longer able to serve as food. A mixture of the extracted yeast and of the extractive was no longer adequate to raise the larvæ, indicating an alteration of the necessary substance by the alcohol. The presence of traces of alcohol in this mixture in itself was not harmful since these flies grow normally in the presence of 2 to 3 per cent alcohol.

The necessary substance in yeast cannot be salts, or cane or grape sugar since non-sterile flies grow normally on Pasteur media, as Loeb's previous experiments have shown.

The addition of butter, nucleic acid, thymus or thyroid extract to the synthetic culture media mentioned in the table was also without effect. This fact, as well as the fact that the flies will not develop on sterile milk, shows that the necessary substance must be different from that needed for pigeons, rats, and other warm-blooded animals.

These observations show that an apparently polyphagous insect like *Drosophila* is in reality a monophagous insect, as yeast is a sufficient and perhaps the only sufficient food for them. They can live on any culture medium which can serve as a food for yeast with which they probably infect it. The experiments show that in the discussion of the synthetic power of the higher animals, the possible action of the microorganisms in the intestine must also be considered. As far as the origin of insects like *Drosophila* is concerned, their existence depends primarily on yeast.

THE INFLUENCE OF PARTURITION ON THE COMPOSITION AND PROPERTIES OF THE MILK AND MILK FAT OF THE COW.*

By C. H. ECKLES and LEROY S. PALMER.

(*From the Department of Dairy Husbandry, University of Missouri, Columbia.*)

(Received for publication, September 6, 1916.)

INTRODUCTION.

The question of variations in the composition of milk is a twofold one; one phase deals with variations in milk as secreted by the cow; the other phase deals with the changes in composition after the milk is secreted and before it is used for food. The latter phase of the question has in recent years overshadowed in large measure the question of the relation between the normal variation in composition to which milk is subject and its use as human food. The chemical side of this question has been for several years the object of critical study by the Missouri Agricultural Experiment Station. The results in regard to a number of the factors involved have already been published.[1]

* Published by permission of the Director of the Missouri Agricultural Experiment Station. The data reported in this paper were collected in cooperation with the Dairy Division, United States Department of Agriculture, Bureau of Animal Industry. While the greater part of the data were taken the Dairy Division was represented by Leroy S. Palmer, who has been responsible for the preparation of the present paper.

[1] Eckles, C. H., and Shaw, R. H., Influence of the stage of lactation on the composition and properties of milk, *U. S. Dept. Agric., Bureau of Animal Industry, Bull. 155*, 1913; Influence of breed and individuality on composition and properties of milk, *ibid., Bull. 156*, 1913; Variations in composition and properties of milk from the individual cow, *ibid., Bull. 157*, 1913. Eckles, C. H., Influence of fatness of cow at parturition on per cent of fat in milk, *Missouri Agric. Exp. Station, Bull. 100*, 1912. Palmer, L. S., and Eckles, C. H., Carotin—the principal natural yellow pigment of milk fat, *J. Biol. Chem.*, 1914, xvii, 191, 211, 223, 237, 245; also *Missouri Agric. Exp. Station, Research Bull. 9, 10, 11*, and *12*, 1914. Eckles, C. H., and Palmer, L. S., Influence of plane of nutrition of cow upon composition

Investigations of other factors of importance influencing the normal composition of cows' milk are still in progress.

The general opinion prevails that cows' milk is not suitable for human food for a period of time after parturition. Opinion varies as to the length of time the milk is unfit for use, as low as 2 days and as high as 15 days being stated as the proper period by different authorities. The methods and standards for certified milk adopted by the American Association of Medical Commissions[2] place the figure at 7 days after parturition. The basis for the exclusion of cows' milk immediately after the birth of the calf is that its composition or constituents give rise to intestinal disorders. The evidence upon which this conclusion is based is, however, extremely difficult to find. The literature on colostrum milk is extensive,[3] but it is astonishingly free from specific data regarding the healthfulness or unhealthfulness of cows' milk immediately after parturition.

The data which are offered in the present paper indicate that colostrum milk is subject to variations in composition, or in other words that the effect of parturition on the composition of cows' milk may be greatly influenced by other factors. Data are presented on two questions: (1) the effect of milking the cow up to the time of parturition; (2) the influence of the length of the period the cow is dry before parturition.

Effect of Milking until Parturition, on Composition of Colostrum Milk and Milk Fat.

Milking the cow up to the time of parturition is not a common practice among dairymen. It is believed by these men that the dairy cow will produce more milk during the lactation period if given a rest of several weeks before parturition. Continuous

and properties of milk and butter fat. Influence of overfeeding, *ibid.*, *Research Bull. 24*, 1914; Influence of underfeeding, *ibid.*, *Research Bull. 25*, 1916.

[2] *Proc. Sixth Ann. Conference, Am. Assn. Med. Milk Commissions*, 1912, 124.

[3] The literature on colostrum is reviewed very thoroughly by Weber, E., Untersuchungen über die Kolostralmilch der Kuh, der Ziege und des Schafes, *Milchwirt. Zentr.*, 1910, vi, pt. x, 433, pt. xi, 481, pt. xii, 543; also by Engel, St., Die Biochemie des Kolostrums, *Ergebn. Physiol.*, 1911, xi, 41–103.

milking of the cow is, however, a common practice in many localities. The effect of this practice on the composition of the colostrum is thus a matter of considerable interest, practical as well as scientific.

HISTORICAL.

We have been able to find only two references in regard to the influence of continuous milking on the colostrum milk. Nasse[4] many years ago stated that the change from colostrum to normal milk occurs very rapidly in the case of cows milked up to the time of parturition. Cook and Hills[5] reported the composition of the milk of a Jersey cow the night before and also a few hours after calving. The results as given by these authors are shown in Table I.

TABLE I.

*Composition of Milk before and after Calving.**

	Fat.	Protein.	Sugar.
	per cent	*per cent*	*per cent*
Before calving	10.18	6.35	4.92
After calving	7.48	6.48	5.18

* Cook and Hills.

The authors state that, "The cow instead of giving colostrum gave milk. . . . Under the microscope but few colostrum particles were found in the first milk." It is doubtful if the conclusion is justified that the milk after calving was normal, inasmuch as a protein percentage of 6.48 is abnormally high. The first milking was unquestionably less abnormal, however, than usually found for colostrum milk.

EXPERIMENTAL.

The data presented in the present paper were taken from three pure-bred Jersey cows in the University herd. Complete analyses of the milk and milk fat were made on composite samples taken at suitable intervals and covering a suitable length of time for a period both before and after parturition. The length of time covered by the composites varied, as indicated in each case in connection with the data.

[4] Nasse, *Arch. Anat. Physiol. u. Wiss. Med.*, 1840, 259, cited by Weber, *Milchwirt. Zentr.*, 1910, vi, 440.

[5] Cook, W. W., and Hills, J. L., Abnormal Milks, *Vermont Agric. Exp. Station, 5th Ann. Report*, 1891, iii.

The analyses made included the specific gravity of the milk, the percentage of total solids, ash, fat, lactose, total protein, casein, albumin (heat-coagulable proteins), and in the case of two of the three animals the percentage of residual protein and residual non-protein nitrogen. The analyses of the milk fat included the saponification value, Reichert-Meissl number, iodine absorption value, and melting point.

The methods of analysis were largely those of the Association of Official Agricultural Chemists. In the fat analyses the Reichert-Meissl number was determined by the Leffman-Beam method, the iodine value by the Hübl method, and the melting point by Wiley's method. In the milk analyses the specific gravity was taken with the modified[6] Quevenne lactometer, the total solids were determined by the Babcock asbestos method, the fat in some cases was determined by the Babcock asbestos method but in others by the Babcock centrifugal method, the lactose by the optical method, using acid mercuric nitrate, the total protein and casein by the official A. O. A. C. methods, and the albumin (heat-coagulable proteins) by the provisional method of this Association. The residual protein was determined by the addition of 15 cc. of Almen's tannic acid reagent to the filtrate from the albumin determination. The precipitate was filtered off and treated like the albumin. The nitrogen remaining in the filtrate after the tannic acid precipitation was determined by evaporating the solution and treating in the usual way (Kjeldahl method). The nitrogen so determined was called the residual non-protein nitrogen. In some cases it was determined by difference between the total nitrogen and the total protein nitrogen.

The methods of taking the composites and the preparation of the milk and butter fat for analysis were practically identical with those described in detail elsewhere.[7] In the analyses made in 1913 the amount of formaldehyde used for preservation of the milk was increased from the earlier practice of one part in 5,000 to 2,500 to one part in 1,650.

The cows used in the investigation were in normal health and condition. The length of time since the last parturition was normal for each animal, being 408 days in the case of Cow 317, 375 days in the case of Cow 57, and 319 days in the case of Cow

[6] Shaw, R. H., and Eckles, C. H., The estimation of total solids in milk by the use of formulas, *U. S. Dept. Agric., Bureau of Animal Industry, Bull. 134*, 1911, 16.

[7] Eckles and Shaw, *U. S. Dept. Agric., Bureau of Animal Industry, Bull. 155*, 1913.

4. The character of the rations of the cows was not identical, but was uniform for the individual animals throughout the experimental period. Cows 317 and 57 were on pasture throughout, but received grain in addition, the latter consisting of a mixture of corn 4 parts, bran 2 parts, and linseed meal 1 part. Cow 4 was fed a grain mixture of the same character, but the roughage which she received consisted of corn silage and alfalfa hay.

RESULTS.

The analyses of the samples in the case of the three cows are shown in Tables III, IV, and V.

Before considering the effects of continuous milking on the composition of the colostrum milk in the case of the three cows it is necessary to have in mind the composition of colostrum when the cow is dry for a period of time before parturition; in other words, normal colostrum milk. Unfortunately, for purposes of the present discussion normal colostrum milk is not uniform in composition with different animals. As has been expressed by other authors, the difficulty arises in that the numerous analyses of colostrum milk which have been made have not been uniform with respect to the length of time after parturition the sample was taken for analysis. The reason for the lack of uniformity of colostrum analyses becomes apparent when the rapidity with which milk changes in composition after parturition is taken into consideration.

In general, however, colostrum milk is characterized by abnormally high specific gravity and percentage of total solids, ash, and protein, and by an abnormally low percentage of lactose. The fat varies, as reported by different investigators, some reporting high fat percentages, others normal, and still others low. The distribution of the proteins of normal colostrum appears to be characteristic, all the protein being present in amounts above normal, the chief characteristic being that the heat-coagulable proteins greatly predominate over the casein, which is the reverse of normal cows' milk. It also appears to be well established that the heat-coagulable proteins of colostrum consist largely of globulin, which is present in normal cows' milk in smaller proportion than albumin. The fat of colostrum milk is characterized by a low Reichert-Meissl number and saponification value, and a high iodine value and melting point. Colostrum is also characterized by a high content of epithelial cells, their peculiar grouping in the milk giving rise to the name "colostrum cor-

puscles." Colostrum is also characterized by its high yellow color, which has been found by us[8] to be due merely to a high concentration of carotin, the normal pigment of the milk fat of the cow.

The general characteristics of colostrum as stated in the preceding paragraph are shown in tabular form in Table II, minimum, maximum, and average values being given as found by various investigators.

The data in Tables III, IV, and V show examples of colostrum milk widely different in composition from normal colostrum as given in Table II, the milk from Cows 317 and 57 showing a greater difference than that from Cow 4.

TABLE II.

Composition of Normal Colostrum Milk.

	Minimum.	Maximum.	Average.*
Specific gravity	1.032	1.090	1.068
	per cent	*per cent*	*per cent*
Total solids	16.18	37.21	28.30
Ash	0.85	2.31	1.78
Total protein	5.72	27.33	15.85
Casein	2.64	7.59	4.82
Albumin	0.25	2.00	
Globulin	2.35	17.51	
Heat-coagulable proteins	5.06	20.21	15.85
Lactose	1.02	3.50	2.48
Fat	0.15	12.00	3.37**
Saponification value	209.5	226.4	
Reichert-Meissl number	6.16	26.0	
Iodine value	36.8	46.4	

* The average values are those reported by Eugling, who made analyses from twenty-three cows (*Ber. Tätigkeit landw. Versuchs. Vorarlberg*, 1878, ii, 33).

** Average of 51 analyses made by König (*Chem. mensch. Nahr. u. Genussmittel*, 1904, ii, 603) showed 3.97 per cent fat.

Considering the results in detail we find that parturition failed to exert the usual depressing influence on the percentage of lactose in each of the three cases, although it was somewhat below

[8] Palmer and Eckles, *J. Biol. Chem.*, 1914, xvii, 199.

TABLE III.

Effect of Milking until Parturition, on Composition and Properties of Colostrum Milk and Milk Fat. Cow 317. July 21 to Aug. 26, 1913.

Date.	Milk per day.	Sp. gr.	Total solids.	Ash.	Lactose.	Fat.	Total proteins.	Casein.	Heat-coagulable proteins.	Residual proteins.	Residual* non-protein nitrogen.	Percentage of total protein present as			Fat.			
												Casein.	Heat-coagulable.	Residual.	Saponification value.	Reichert-Meissl No.	Iodine value (Hübl).	Melting point.
1913	*lbs.*	*1.0—*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*							*°C.*
July 21–23	14.1	337	13.34	0.71	4.61	4.23	3.78	2.62	0.81	0.35	0.024	69.3	21.4	9.3	228.5	29.81	34.57	33.20
" 24–26	14.1	335	13.51	0.72	4.68	4.35	3.76	2.70	0.68	0.38	0.058	71.8	18.1	10.2	227.9	27.54	34.87	30.70
" 27–29	13.5	332	13.29	0.72	4.55	4.15	3.87	2.77	0.71	0.39	0.057	71.6	18.4	10.0	226.1	28.59	36.04	31.90
" 30–Aug. 1	12.3	348	13.62	0.73	4.57	4.18	4.14	2.81	0.97	0.36	0.051	67.9	23.4	8.7	226.3	27.17	36.63	32.60
Aug. 2, a.m.**	7.4	373	13.47	0.77	4.51	3.58	4.61	2.86	1.35	0.40	0.055	62.0	29.3	8.7	224.7	27.11	37.00	34.50
Parturition at noon.																		
Aug. 2, p.m.	6.5	368	14.01	0.78	4.43	4.00	4.80	2.72	1.70	0.38	0.045	58.8	35.4	5.8	223.0	24.43	37.09	34.10
" 3	15.5	367	12.22	0.80	4.31	2.78	4.33	2.82	1.13	0.38	0.064	65.1	26.1	8.8	221.1	22.50	38.14	34.80
" 5	18.1	331	13.61	0.82	4.39	4.20	3.77	2.74	0.63	0.40	0.065	72.7	16.7	10.6	220.3	23.83	40.17	34.30
" 7	23.4	328	13.86	0.75	4.48	4.60	3.71	2.75	0.56	0.40	0.050	74.2	15.1	10.7	223.3	25.14	38.34	37.10
" 9	21.5	319	12.65	0.76	4.20	3.95	3.41	2.54	0.43	0.44	0.042	74.5	12.6	12.9	222.6	26.52	37.68	36.80
" 11–12	21.5	319	12.38	0.75	4.40	3.70	3.28	2.46	0.44	0.38	0.039	75.0	13.4	11.6	222.4	27.74	36.71	35.40
" 13–14	22.1	318	12.48	0.74	4.44	3.80	3.22	2.45	0.39	0.38	0.044	76.1	12.1	11.8	222.7	26.55	38.29	37.20
" 15–16	21.9	316	12.47	0.71	4.40	3.95	3.18	2.36	0.44	0.38	0.038	74.2	13.8	12.0	227.7	26.55	38.46	36.90
" 17–18	24.3	317	12.38	0.71	4.47	3.75	3.18	2.45	0.37	0.36	0.042	77.0	11.6	11.4	224.9	29.47	36.42	34.90
" 25–26	25.6	314	11.98	0.70	4.43	3.60	3.00	2.29	0.38	0.33	0.040	76.3	12.7	11.0	226.8	30.42	34.43	35.20

* Difference between total nitrogen and total protein nitrogen.

** Length of lactation period ending Aug. 2, 408 days.

TABLE IV.

Effect of Milking until Parturition, on Composition and Properties of Colostrum Milk and Milk Fat. Cow 57. July 12 to Sept. 3, 1913.

Date.	Milk per day.	Sp. gr.	Total solids.	Ash.	Lactose.	Fat.	Total proteins.	Casein.	Heat-coagulable proteins.	Residual proteins.	Residual* non-protein nitrogen.	Percentage of total protein present as			Fat.			
												Casein.	Heat-coagulable.	Residual.	Saponification value.	Reichert-Meissl No.	Iodine value (Hübl).	Melting point.
1913	*lbs.*	1.0—	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*							*°C.*
July 12–14	5.1	338	15.45	0.80	4.50	4.90	4.89	3.37	1.11	0.41	0.063	68.9	22.7	8.4	220.6	25.60	36.96	37.2
" 15–17	5.2	304	15.02	0.80	4.38	4.63	4.92	3.37	1.18	0.37	0.047	68.5	24.0	7.5	223.1	27.62	35.77	37.6
" 18–20	6.1	342	14.61	0.81	4.60	4.10	4.76	3.27	1.10	0.39	0.054	68.7	23.1	8.2	225.7	28.62	35.08	37.3
" 21–23	6.3	366	14.75	0.80	4.60	4.40	4.67	3.28	1.01	0.38	0.044	70.2	21.6	8.2	226.8	29.58	34.43	37.7
" 24–26	5.9	367	14.97	0.79	4.78	4.55	4.54	3.15	1.00	0.39	0.048	69.4	22.0	8.6	226.0	29.92	34.91	37.9
" 27–29	5.8	352	14.94	0.77	4.55	4.55	4.70	3.20	1.10	0.40	0.059	68.1	23.4	8.5	225.3	29.27	35.64	38.2
" 31–Aug. 1	5.4	362	14.79	0.80	4.34	4.50	4.85	3.21	1.21	0.43	0.048	66.1	25.0	8.9	225.5	29.42	35.87	38.3
Aug. 2– 4	5.2	368	14.61	0.76	4.38	4.22	4.87	3.14	1.31	0.42	0.060	64.5	26.9	8.6	226.2	27.91	36.50	37.0
" 5– 7	6.1	370	14.41	0.76	4.36	3.98	4.86	3.05	1.37	0.44	0.072	62.8	28.2	9.0	224.5	27.72	35.10	35.0
" 8–10	7.3	376	14.54	0.75	4.76	3.75	4.90	3.06	1.46	0.38	0.061	62.5	29.8	7.7	225.1	27.25	35.81	35.8
" 11, a.m.**	4.5	380	13.56	0.80	4.29	2.78	5.26	3.02	1.82	0.42	0.068	57.4	34.6	8.0				
Parturition at noon.																		
Aug. 11, p.m.	3.9	380	14.86	0.80	4.62	3.78	5.18	3.04	1.81	0.33	0.078	58.7	34.9	6.4				
" 12	17.2	378	13.28	0.77	5.06	2.65	4.32	3.06	0.91	0.35	0.075	70.8	21.0	8.1	227.9	27.23	35.05	33.3
" 13	26.4	356	13.25	0.79	4.72	3.35	4.21	3.02	0.81	0.38	0.039	71.8	19.2	9.0	227.0	27.28	34.45	32.8
" 15	23.6	350	13.19	0.77	5.10	4.17	3.88	3.10	0.36	0.42	0.042	79.9	9.3	10.8	226.7	31.17	37.09	35.8
" 18	27.7	342	13.63	0.76	5.10	3.85	3.65	2.90	0.32	0.43	0.048	79.5	8.8	11.8	229.7	34.89	35.87	35.0
" 20	25.3	329	13.94	0.72	5.10	4.58	3.27	2.60	0.31	0.36	0.041	79.5	9.5	11.0	224.6	30.76	37.76	36.0
" 22–23	26.9	334	14.37	0.73	5.16	5.00	3.32	2.72	0.19	0.41	0.023	81.9	5.7	12.4	224.3	31.61	40.04	34.9
" 24–25	27.4	333	14.08	0.73	5.19	4.60	3.34	2.71	0.22	0.41	0.033	81.1	6.6	12.3	226.7	32.01	39.36	33.8
" 26–27	27.2	333	14.08	0.71	5.15	4.60	3.37	2.67	0.17	0.53	0.022	79.3	5.0	15.7	225.7	31.45	38.22	34.1
Sept. 2– 3	26.6	322	13.86	0.68	5.16	4.58	3.23	2.25	0.34	0.64	0.030	69.7	10.5	19.8	228.5	30.59	35.40	31.8

* Difference between total nitrogen and total protein nitrogen.

** Length of lactation period ending July 11, 375 days.

TABLE V.

Effect of Milking until Parturition, on Composition and Properties of Colostrum Milk and Milk Fat. Cow 4.

* Difference between total nitrogen and total protein nitrogen.
** Length of lactation period ending July 11, 375 days.

TABLE V.

Effect of Milking until Parturition, on Composition and Properties of Colostrum Milk and Milk Fat. Cow 4. Sept. 17 to Oct. 12, 1907.

Date.	Milk per day.	Sp. gr.	Total solids.	Ash.	Lactose.	Fat.	Total proteins.*	Casein.	Heat-coagulable proteins.	Percentage of total protein present as		Fat.			
										Casein.	Heat-coagulable.	Saponification value.	Reichert-Meissl No.	Iodine value (Hübl.)	Melting point.
1907	*lbs.*	*1.0—*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*						*°C.*
Sept. 17.....	12.1	346	15.83		4.50	5.36	4.85	3.70	0.70	76.3	14.4	231.4	23.97	32.43	32.70
" 20.....	8.6	362	17.77		4.68	6.38	5.42	4.40	0.83	81.1	15.3	234.1	25.69	32.87	32.85
" 23.....	11.3	360	16.94		4.79	6.04	5.17	3.95	0.89	77.9	17.2	231.3	25.28	33.51	33.20
" 26.....	10.7	376	17.58		5.04	5.96	5.68	4.27	0.83	76.6	14.6		13.88	30.39	34.35
" 29.....	9.9	390	18.11		4.64	5.82	6.19	4.66	1.02	75.3	16.5	217.4	10.75	36.16	34.05
Oct. 2.....	9.9	420	19.16		3.95	6.30	7.02						8.90	32.03	37.03
" 4**.....	11.3	445	18.48		4.30	5.90	6.57	4.85	0.96	73.8	14.4				
Parturition.															
Oct. 5 a.m...	13.8	330	20.88	0.87	4.59	7.20	7.02	4.47	0.89	63.7	12.7				
p.m...		330	20.32			8.50	6.51	4.34	0.70	66.7	10.8	221.3	19.14	30.02	38.13
" 6 a.m...	22.3	330	15.92			5.00	5.74	4.34	0.51	75.6	8.9	222.1	17.76	31.13	37.33
p.m...		310	16.36		4.80	6.06	5.10	3.64	0.43	71.4	8.4	224.1	18.57	33.18	36.35
" 7 a.m...	27.7	310	16.32	0.74	5.71	6.05	4.72	3.83	0.34	81.1	7.2	222.7	19.96	31.32	36.03
p.m...		340	16.87		5.35	6.25	4.53	3.38	0.61	74.6	13.5	224.9	22.13	31.30	36.18
" 8 a.m...	26.3	335	15.82	0.78	5.72	5.36	4.91	3.77	0.50	76.8	10.2	224.3	22.76	32.69	35.25
p.m...		350	15.25	0.81	5.22	5.32	4.59	3.57	0.54	77.8	11.8	225.0	23.19	33.21	35.50
" 9 a.m...	30.1	355	14.84	0.79	5.48	4.89	4.40	3.45	0.53	78.4	12.1	228.1	24.13	32.26	34.60
p.m...		325	15.33	0.82	5.35	5.31	4.20	3.32	0.51	79.1	12.1	226.2	23.36	33.51	34.65
" 10 a.m...	29.7	340	14.84	0.76	5.53	4.83	4.40	3.38	0.59	76.8	13.4	224.8	25.29	33.76	34.48
p.m...		335			5.65							226.5	24.37	34.62	34.45
" 11 a.m...	28.6	340	14.96	0.74	5.67	5.16	4.47	3.13	0.42	70.1	9.4	225.3	25.21		33.70
p.m...		350	13.92	0.81	5.35	4.31	4.21	3.06	0.43	72.8	10.2	220.6	24.23	34.84	33.08
" 12 ...	31.2	335	14.28	0.78	5.42	4.60	4.15	3.00	0.43	72.4	10.4	228.2	24.68	33.92	33.68

* Total N × 6.38.

** Length of lactation period ending Oct. 4, 319 days.

normal in the case of Cows 57 and 4. In regard to the casein we find no effect in the case of Cows 317 and 57, but a high casein percentage for Cow 4. The composition of the milk fat was uniformly unaffected by parturition in the case of each of the three cows. Very abnormal fat constants followed parturition in the case of Cow 4, but it is open to question whether this was the result of parturition, inasmuch as the fat showed the same abnormalities for several days before parturition in even more striking degree.

The most uniform effect of parturition was upon the heat-coagulable proteins; namely, the albumin and globulin of the milk. The percentage of these proteins increased very materially up to the time of parturition, reaching a maximum in the first milk drawn after parturition. The proportion of the total protein of the milk attained at this time was remarkably great for Cows 317 and 57, being nearly 35 per cent in each case. In spite of this great increase in albumin and globulin the actual proportion of the total milk represented by these proteins was far below the minimum value for heat-coagulable proteins of normal colostrum as given in Table II.

As already pointed out, the results in the case of Cow 4 were less striking than for the other two cows. The authors attribute this to the fact that the milk and milk fat of this cow showed the characteristics of the end of the lactation period before parturition occurred.

The general conclusion which we believe is justified by the data given for the three cows under discussion is that when a cow is milked up to parturition the colostrum milk will follow closely the composition of the milk given before parturition, with the exception of a more or less marked increase in the heat-coagulable proteins. If the composition of the milk and milk fat shows the characteristics of the end of the lactation period before parturition, the colostrum milk and milk fat will also show these characteristics. If the milk and milk fat are normal in composition before parturition the colostrum milk and milk fat will also be normal, with the exception already noted.

Influence of Length of Period of Dryness of Cow, on Composition of Colostrum Milk and Milk Fat.

The data given in the foregoing pages of this paper indicate clearly that the abnormal composition of milk and milk fat usually characteristic of colostrum is greatly lessened when the cow is milked up to parturition, and may be evident only to a slight degree in the case of a heavy milking cow. This result raises the question whether the length of time the cow is dry before parturition influences the composition of colostrum milk. Winterstein and Strickler[9] have suggested that this factor has a great influence on the properties of colostrum, greater than the influence either of breed or manner of feeding. St. Engel,[3] however, questions whether this viewpoint can be upheld. Neither investigator offered any data in support of his opinion. Certain data which we have collected in connection with our colostrum studies and which seem to have a bearing on this point are given in Table VI. This presents complete analyses of the milk and milk fat for the first two or three milkings of four cows, following parturition. The length of the period of dryness of the cows varied from 19 to 83 days. The animals were all pure-bred dairy cows, on pasture, and probably received some additional grain, which is the usual practice in the dairy herd of the Missouri Agricultural Experiment Station. Our records, however, are not complete on this point in connection with these cows. The methods of analysis, handling of cows, etc., were identical with the procedure described in the first part of the paper in connection with the other data reported.

In the case of each of the animals the first milk was drawn within a few hours after parturition. The animals having been dry for a period of time, the quantity obtained was not sufficient in all cases for complete analyses of both milk and milk fat. Such data as it was possible to secure are reported in the table. Complete analyses were made in each case as soon as sufficient milk was obtained.

[9].Winterstein, E., and Strickler, E., Die chemische Zusammensetzung des Colostrums mit besonderer Berücksichtigung der Eiweissstoffe, *Z. physiol. Chem.*, 1906, xlvii, 58.

TABLE VI.

Influence of Period of Dryness of Cow, on Composition and Properties of Colostrum Milk and Milk Fat.

Cow.	Period dry.	Date.	Milk per day.	Sp. gr.	Total solids.	Ash.	Lactose.	Fat.	Total proteins.	Casein.	Heat-coagulable proteins.	Residual protein.	Residual non-protein nitrogen.	Percentage of total protein as			Fat.			
														Casein.	Heat-coagulable.	Residual.	Saponification value.	Reichert-Meissl No.	Iodine value (Hübl).	Melting point.
	days		*lbs.*	*1.0—*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*							*°C.*
		1913																		
22*.....	19	July 5, a.m.	4.6	345		0.71		4.43	6.64	4.02	1.86	0.76	0.055	60.6	28.0	11.4				
		" 5, p.m.		342	13.08	0.75	4.13	3.95	5.01	3.45	1.07	0.49	0.045	68.8	21.4	9.8				
		" 6	7.0	350	13.09	0.81	4.28	3.10	4.45	3.29	0.75	0.41	0.041	74.0	16.8	9.2	222.2	21.14	33.14	35.9
		1908																		
205**....	20	Aug. 27, a.m.		510	17.44		2.62	2.85	9.95	4.66	4.66			46.8	46.8					
		" 27, p.m.	1.9	372	12.51	0.83	3.60	1.98	6.38	3.77	1.40			59.1	21.9			27.07		35.9
		1913																		
17†.....	30	May 30, a.m.				0.84														
		" 30, p.m.		343	13.72	0.83	4.70	2.80	5.11	3 68	0.94	0.49	0.042	72.0	18.4	9.6				
		" 31	14.7	334	13.17	0.84	4.72	2.63	4.69	3.56	0.66	0.47	0.035	75.9	14.1	10.0	224.6	21.50	34.89	35.8
		1908																		
400‡.....	83	Oct. 23, a.m.		366	22.22	0.80	2.65	1.30	14.42	7.90	5.53			54.7	38.3				30.33	38.6
		" 23, p.m.		345	15.78	0.95	3.81	3.26	7.91	5.30	1.96			67.0	24.8		223.6	21.92	25.65	39.7

* Parturition during night of July 4.

** Parturition during early morning of Aug. 27.

† Parturition during night of May 29.

‡ Parturition during night of Oct. 22.

DISCUSSION OF RESULTS.

Taking into account the fact that the first milking after parturition, that is, the true colostrum, in the case of Cow 17 was not sufficient for any analysis save that of percentage of ash, the data in Table VI show the interesting fact that the abnormalities in composition of colostrum milk are in direct relation to the length of the period of dryness. All the colostrum samples showed a composition more or less characteristic of the first milk drawn after parturition, but this was particularly striking in the case of Cow 400, which was dry 83 days. The percentage of total solids, protein, casein, heat-coagulable proteins, and ash was abnormally high in each case, while the percentage of lactose and fat was abnormally low. However, in the case of Cow 22, dry 19 days, the milk was much less abnormal in composition, although it showed a protein, casein, and heat-coagulable protein content considerably above normal. The fat percentage of the colostrum milk of Cow 17, namely 4.43, which was only slightly below the normal fat percentage of this cows' milk (which was 4.8 per cent) is in striking contrast to the extremely low fat percentage of the colostrum milk of Cow 400.

The data in Table VI are less complete in regard to the physical and chemical constants of the colostrum fat in the case of the four cows, owing to the fact, already mentioned, that there was insufficient milk to furnish enough fat for these analyses. The data available indicate, however, that the length of the period of dryness had little, if any, influence on the composition of the colostrum fat. In each case the fat showed the composition characteristic of colostrum fat, having a low saponification and Reichert-Meissl value and high melting point. The iodine value of the fats analyzed did not show the extremely high figure usually characteristic of colostrum fat.

The data in Table VI in general lead to the conclusion that the length of the period of dryness is a factor influencing the composition of colostrum milk, but does not influence the composition of colostrum milk fat. The shorter the time the cow is dry before parturition the less abnormal will be the colostrum milk.

SUMMARY.

The conclusions which we have drawn from the data presented in the present paper may be summarized as follows:

1. Parturition in the case of the cow is normally accompanied by the production of milk of extremely abnormal composition, called colostrum.

2. When cows are milked up to parturition, however, the colostrum milk and milk fat are much less abnormal in composition and follow closely the composition of the milk and milk fat given before parturition.

3. The chief characteristic of the milk as the cow approaches parturition in these cases is the marked increase in the content of heat-coagulable proteins, which reach their maximum in the first milk following parturition, that is, the true colostrum milk. This would indicate that a high content of heat-coagulable proteins is the chief, if not the only, real effect of parturition on the milk.

4. The length of time the cow is dry before parturition is a factor influencing the composition of colostrum milk, but does not influence the composition of colostrum milk fat. The shorter the time the cow is dry the less abnormal will be the composition of the colostrum milk.

A METHOD FOR THE DETERMINATION OF NITRIC NITROGEN.*

By F. M. SCALES.

(From the Laboratories of the Soil Bacteriology Division, Bureau of Plant Industry, United States Department of Agriculture, Washington.)

(Received for publication, September 15, 1916.)

A simple and accurate method of determining nitric nitrogen in culture media and soil extracts has been much needed by soil biologists and chemists. A procedure to be satisfactory must yield reliable results in the presence of unstable organic substances like the amino and amide compounds.

Practically all of the methods now in use involve the reduction of the nitrate to ammonia and its subsequent distillation into standard acid. The reduction is effected by nascent hydrogen which is generated either in an acid or an alkaline solution. The principal objection to the methods using an acid solution is that they take too long to effect reduction, or if the time is shortened by using larger quantities of reagents, the increased amount of acid may hydrolyze some organic material. The better planned methods using an alkaline solution accomplish the reduction during distillation so that much less time is needed to obtain results, but the amount of alkali required is so great that unstable organic matter may be decomposed.

Organic material does not affect the results obtained by the proposed procedure. The method is accurate and requires only the simplest modification of the regular Kjeldahl apparatus. A zinc-copper couple is prepared in such a way that it can be renewed very easily. This couple decomposes water at the boiling point. The hydrogen is evolved and the oxygen of the water combines with the zinc to form zinc oxide. The advantage in the use of this

* Published by permission of the Secretary of Agriculture.

Thanks are due Mr. F. L. Goll of the Office of Soil Bacteriology for the illustration accompanying this report.

couple is that neither an acid nor an alkaline reaction is necessary to promote the generation of hydrogen. Therefore only a small amount of fixed alkali is required to expel the ammonia formed by the reduction.

HISTORICAL.

The fact that a zinc-copper couple has the power of decomposing pure distilled water at room temperature has not been generally known and this undoubtedly is the reason that a direct distillation method using this couple has not been perfected for the determination of nitrates in soil analyses.

M. W. Williams[1] showed that dilute solutions of nitrate are completely reduced to ammonia by this couple if kept at 30°C. for a few hours, and that the reduction is hastened by the presence of carbonic acid, and a neutral electrolyte like sodium chloride. He poured the solution containing the ammonia into a Nessler tube and titrated with Nessler's reagent in the ordinary way.

Keating Stock[2] used granulated zinc for his couple and reduced the nitrate solution at 20–25°C. for 48 hours. He distilled off the ammonia and Nesslerized. This method is slower than most of the acid methods, but it does not take any longer than the acetic acid method with this same couple that is used at the Rothamsted Station.[3]

Apparatus.

The apparatus designed for use with this method is the simplest one that will satisfactorily scrub the distillate before the ammonia formed by the reduction passes into the standard acid. The apparatus may be understood by referring to Fig. 1.

The receiving end of the Wagner form of connection tube is fitted with an extra long rubber stopper[4] (A) which is slipped almost up to the bulb.

---

[1] Williams, M. W., *Tr. Chem. Soc.*, 1881, xxxix, 100; *Analyst*, 1881, vi, 36.

[2] Stock, K., cited in Sutton, F., Volumetric Analysis, Philadelphia, 10th edition, 1911, 468.

[3] Russell, E. J., *J. Agric. Sc.*, 1914, vi, 18.

[4] A. H. Thomas Co.'s No. 25 (28 mm. bottom by 34 mm. top and 38 mm. deep).

A large size lipless test-tube (B) about 23 mm. in diameter by 150 mm. in length is fitted with a rubber stopper (C) containing two holes, one for the receiving end of the connection tube, and the other for the glass tube (D) which goes to within 1 cm. of the bottom of the test-tube. The holes in the rubber stopper should be made rather small for the tubes to insure a tight joint. If the tubes are heated a little before they are finally put

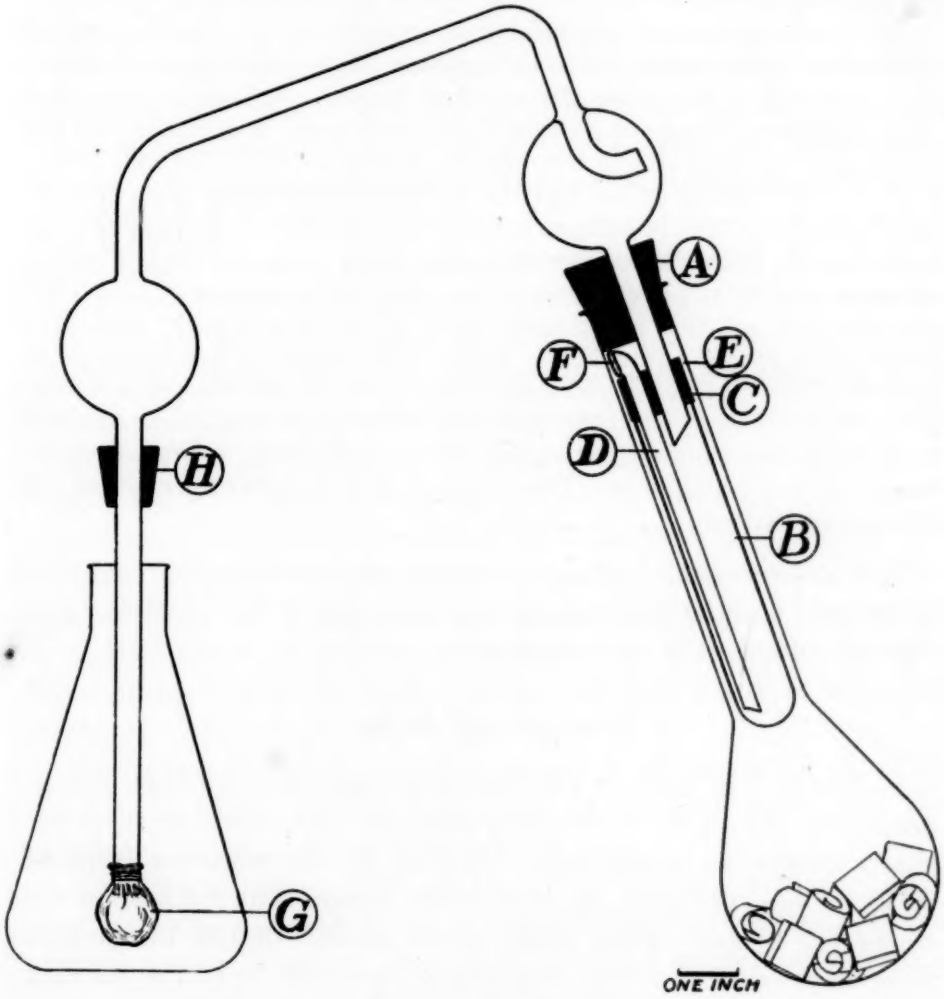


FIG. 1. Apparatus for the determination of nitric nitrogen with the zinc-copper couple.

in position they will stick firmly to the stopper. If the bottom of the stopper when pushed as far as possible into the test-tube does not press firmly against the sides of the tube it must be cut off to the place where its sides do press against the tube. When steam gets between the test-tube and the stopper it will soon work to the top and the stopper will have to be replaced. The joint (E) between the test-tube and the rubber

stopper is covered with half inch electric tape which is wound twice around. This part of the apparatus should be made so that the sides of the test-tube will be a uniform distance of 2 or 3 mm. from the neck of the distilling flask.

The receiving end (F) of the glass tube (D) may be made by sealing the end of the tube and then blowing a large hole in the side just below the seal.

The glass perforated spherical enlargements on the delivery end of distillation tubes insure better absorption in the acid solution than a plain tube but a still more efficient bulb for this purpose is easily made of cambric[5] by cutting out a circle of the cloth 9 cm. in diameter and then gathering it in folds around the delivery end of the tube (G), and tying it with a cotton string which like the cloth has previously been boiled in distilled water. The bag thus formed should measure, when pulled down, about 3.5 cm. from the bottom up to the string. All the cloth above the string should be cut down close to it. The entire surface of this bag in the receiving solution being acid, much of the ammonia is neutralized before it is forced through the cloth. The steam comes through the surface of the cloth in a uniformly fine spray till the receiving solution becomes hot enough when the size of the bubbles increase, but under ordinary conditions they do not exceed 1 mm. in diameter. Bags thus prepared will last a long time. Those used in this work have been in at least 500 determinations.

The writer usually places a rubber stopper (H) just under the glass bulb to facilitate raising the tube out of the receiving solution at the end of the distillation.

Solutions and Reagents.

Indicator.—A test of various indicators showed that *p*-nitrophenol is the most satisfactory one for this work as it gives a sharp end-point in solutions ranging in temperature from 37–70°C. The solutions at the lower temperature stood in cold water for a short time while those at the higher temperature were titrated 5 minutes after being removed from the distilling shelf. The indicator is prepared by dissolving 1 gm. of the commercially pure substance in 100 cc. of 50 per cent alcohol.

Alkaline Solution.—The $\frac{N}{14}$ alkaline solution was prepared from Merck's sticks of potassium hydroxide purified by alcohol, and standardized against chemically pure benzoic acid.[6]

[5] Cambric 100 (Berkeley Mills). The weight of 100 sq. cm. of this material unboiled is 0.8 gm.

[6] The c.p. benzoic acid was obtained from the Bureau of Standards. Their directions were followed with regard to the standardization.

Acid Solution.—The sulfuric acid solution was made exactly equal in strength to the potassium hydroxide solution.

Ammonia-Free Water.—The water used for all this work was redistilled after acidifying with sulfuric acid to remove ammonia.

Sodium Nitrate Solution.—The sodium nitrate standard was prepared by neutralizing titrated nitric acid solution with sodium hydroxide.

Zinc.—The zinc used for the couples is ordinary sheet zinc about 0.5 mm. thick. It is cut in strips 2 cm. wide by 8 cm. long. These strips are coiled by rolling around a pencil and then opened so that the outermost circle of the coil is approximately 2 cm. in diameter. A coil weighs about 5 gm. 80 gm., or sixteen of these coils, are dropped into each of the flasks. There ought to be about 200 cc. of water in the flask so that the coils will not directly strike the glass as they fall in. The coils may be left in the flasks and used indefinitely if their weight is maintained at 80 gm. by adding one new coil about every week when they are used continually. The flask holders of Pickel[7] are very useful for the flasks containing the coils. In using zinc for the first time it should be treated with an approximately 1 per cent sodium hydroxide solution and then with a 1 per cent sulfuric acid solution for 3 or 4 minutes till it has been well cleaned and a fresh surface of the metal is exposed. The acid solution is then drained from the flask and the couple is ready to be treated with the acid copper sulfate solution.

Acid Copper Sulfate.—This solution is prepared by dissolving 10 gm. of $CuSO_4 . 5H_2O$ in 2 liters of distilled water and then adding 6 cc. of concentrated sulfuric acid.

Alumina Cream.—This suspension is made by dissolving 50 gm. of $Al_2(SO_4)_3 . 18H_2O$ in 2 liters of distilled water, and then adding a few drops of some indicator and neutralizing with an approximately normal sodium hydroxide solution. The solution is well stirred and the precipitate allowed to settle, when the supernatant liquid may be siphoned off and more distilled water added. The precipitate is then allowed to settle over night and the supernatant liquid again siphoned off. The thick cream is kept for clarifying soil extracts.

[7] Pickel, J. M., *J. Ind. and Eng. Chem.*, 1915, vii, 787.

Magnesia.—The Baker and Adamson Chemical Company's heavy magnesium oxide c.p. was used for all determinations.

Sodium Chloride.—A c.p. salt was used for the electrolyte.

PROCEDURE.

The procedure for making a determination is simple and will lend itself readily to routine work. 150 cc. of the acid copper sulfate solution are poured into the flask containing the zinc and allowed to stand at least 8 minutes. The solution may be left in contact with the zinc while the soil extracts are being filtered. It is then poured off and the couple washed once with water. Tap water may be used if it is all poured out again.

The soil extract is prepared by adding 300 cc. of ammonia-free water to 100 gm. of air-dry soil, or if the soil is high in organic matter 25 cc. of alumina cream and 275 cc. of water. This is allowed to stand half an hour with occasional shaking and is then filtered. 200 cc. of the soil extract is poured into the flask and a measureful of a mixture of 5 gm. of sodium chloride and 1 gm. of magnesia emptied into it. The rubber stopper of the connection tube is fitted into the mouth of the flask, and the delivery end of the connecting tube is placed in the receiving flask, which contains the $\frac{N}{14}$ acid and sufficient distilled water to bring the volume to 100 cc. The gas is lighted and the flame adjusted so that the contents of the flask will boil in 9 to 12 minutes. The boiling is continued for 45 minutes, during which time at least 125 cc. of the soil extract should be distilled over. The receiving flask is then lowered and the cambric bag washed with only 1 or 2 cc. of distilled water, so that no vacuum will be formed in the connection tube. When the tube has been slightly cooled by this small addition of water more may be thrown onto the bag where it is tied to the glass tube. This is the only part that it is necessary to wash as the steam will clean out the bag proper. The distillate is allowed to stand about 5 minutes after the distillation is finished so that the temperature will be between 37 and 70°C., when five drops of the *p*-nitrophenol are added and the titration is made with the $\frac{N}{14}$ caustic potash.

It sometimes happens that when the distillation is run too slowly the scrubbing tube becomes almost filled with water.

This condition may be remedied by turning out the gas and as soon as a little of the receiving solution has been drawn up into the connecting tube to form a seal, lowering the receiving flask. The vacuum produced by the cooling of the distillation flask will soon cause the contents of the scrubbing tube to be siphoned over. The gas may then be lighted and the distillation started again.

DATA.

As high organic matter in the solution containing nitrate generally interferes with the accuracy of the determination when made by direct reduction methods, it seemed desirable to find some simple way of removing the bulk of soluble organic matter, so as to eliminate as far as possible any interference by this material with the free generation of hydrogen.

After trying a number of substances it was found that alumina cream would clarify the extract and remove sufficient organic matter to permit a perfect working of the method.

To test the method under rather extreme conditions an extract was prepared from rotted manure by adding 500 cc. of distilled water to 50 gm. of the well dried material. The suspension was frequently shaken and after standing 2 hours was filtered.

15 cc. of this very dark brown solution were added to each of five carefully prepared 100 gm. samples of air-dry greenhouse soil. 25 cc. of alumina cream were added to each sample and in addition 260 cc. of distilled water. Each sample was well shaken and after standing half an hour was poured onto a filter; the first runnings were poured back and an almost perfectly clear and colorless filtrate was obtained. 200 cc. of each of the filtrates were poured into Kjeldahl flasks, 1 gm. of magnesia was added, and the extract boiled to half its volume to expel ammonia. When this was completed the solution, after standing a short time, was cooled by the addition of 50 cc. of redistilled water and then poured onto the couple, the flask was rinsed with approximately 35 cc. of distilled water and then 14.55 mg. of nitric nitrogen were added. The nitrate was not added till just before beginning the distillation in order to eliminate any errors that might arise from manipulation since the test was made to determine whether distillation with these reagents would yield accurate results in the presence of organic matter.

5 gm. of sodium chloride and about 0.5 gm. of magnesia were added to each solution. The extra amount of magnesia was put in to make up for a small quantity lost in transferring the solutions. The flasks were then connected with the rest of the apparatus and the distillation was started. The receiving flasks for the distillates from the soil and manure extracts

contained 25 cc. of standard acid and those for the distillates from the extracts with added nitrate 45 cc. of acid.

A second set of determinations, Nos. 6 to 10, were made with a slightly stronger manure extract and a larger quantity of added nitrate. The titration of the distillates gave the following results.

TABLE I.

Nitric Nitrogen Recovered from Soil and Manure Extract.

Source of extract.	Nitric nitrogen.			
	Added.	Theoretical total.	Found.	Average.
	mg.	*mg.*	*mg.*	*mg.*
1. Soil and manure.....	0.0	10.85	10.9	
2. " " "	0.0	10.85	10.8	10.85
3. " " "	14.55	25.40	25.15	
4. " " "	14.55	25.40	25.35	
5. " " "	14.55	25.40	25.45	25.31
6. " " "	0.0	11.93	12.00	
7. " " "	0.0	11.93	11.85	11.93
8. " " "	15.2	27.13	26.95	
9. " " "	15.2	27.13	27.05	
10. " " "	15.2	27.13	27.15	27.05

The superiority of this method of distilling from a solution made alkaline with only 1 gm. of magnesia is readily apparent.

Allen,[8] appreciating its advantage says: "A very large amount of effort was spent in this work in an attempt to obtain a reliable and accurate method by the reduction in the presence of MgO and distillation with an ordinary Kjeldahl rack, a method which would be extremely simple and rapid."

The low alkalinity produced by this quantity of oxide practically eliminates any possibility of breaking down organic material in the soil extracts.

To test the action of this alkali on organic material duplicate determinations were run with distilled water to which had been added 10 mg. of nitric nitrogen and approximately 10 mg. of nitrogen as urea, sodium asparaginate, glycocoll, peptone, or dried blood. The averages of the duplicate determinations are given in Table II.

[8] Allen, E. R., *J. Ind. and Eng. Chem.*, 1915, vii, 521.

TABLE II.

Nitric Nitrogen Recovered without Hydrolysis of Organic Substances.

Substance.	Organic nitrogen. Quantity.		Nitrogen.		
	Material.	Nitrogen.	Nitric quantity.	Total.	Recovered.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Urea	21	9.79	10.0	19.79	9.95
Sodium asparaginate	122	9.88	10.0	19.88	9.93
Glycocoll	54	10.04	10.0	20.04	9.94
Peptone	66	9.78	10.0	19.78	9.92
Dried blood	91	12.79	10.0	22.79	9.99

A determination made with a solution containing only urea yielded only the small quantity of ammonia contained in the distilled water.

Probable Error.

As the results obtained with this method were accurate it seemed desirable to ascertain the probable error occurring under

TABLE III.

The Probable Error of a Nitric Nitrogen Determination.

	Nitric nitrogen recovered.	
Theoretical value	3.00	25.00
Experiment No.		
	mg.	*mg.*
1	3.00	24.90
2	2.98	24.90
3	3.01	25.00
4	2.98	24.80
5	3.03	24.90
6	3.00	25.00
7	2.95	24.80
8	3.00	25.10
9	3.02	24.80
10	2.97	24.92
11	2.97	24.95
12	2.90	24.96
Average	2.98	24.92
Deviation from theoretical value	−0.02	−0.08
Probable error	±0.007	±0.044

the regular procedure. Freshly prepared couples were accordingly made for twelve determinations with 3 mg. and twelve with 25 mg. of nitric nitrogen and the probable error of a single determination calculated from the results obtained by the method of least squares.

The low probable error shows that the method may be employed where the most accurate results are desired.

DISCUSSION.

The author has found that some couples will yield good results without renewing them for four distillations when there is as much as 25 mg. of nitric nitrogen to be reduced. Such results depend on the age of the zinc, the quality of the couple formed, and possibly other factors. Because of the complexity of this condition the author does not recommend the repeated use of a couple where more than 5 mg. of nitric nitrogen are to be determined and even then if it is a good couple it should not be used for more than four determinations. For the present, until some method is found either for improving or for estimating the condition of a couple, it should be renewed before each determination. When the couple is to be employed without recoppering it should be washed once and used as soon as possible after distillation is finished. The couple should not be left standing in the residue of a distillation over night for the magnesia cakes around the zinc which is then very difficult to clean even by the acid treatment. If the zinc is washed shortly after a distillation a good couple can be made simply by adding the required amount of acid copper sulfate solution. It is, however, well to treat the zinc with 1 per cent sulfuric acid solution every 3 or 4 days to insure the formation of a good couple.

If the solution containing the nitrate is strongly alkaline it should be acidified with sulfuric acid and an excess of magnesia added because strong alkalinity interferes with the generation of hydrogen by this couple.

SUMMARY.

1. A new method for the determination of nitric nitrogen is described. A zinc-copper couple will reduce a nitrate in solution

at the boiling point; therefore the reduction proceeds during the distillation. 2. A simple apparatus has been made to give excellent results with this method. 3. Accurate results are obtained with solutions high in organic matter if they are clarified with a small quantity of alumina cream. 4. Unstable organic matter is not broken down by this procedure, owing to the slight alkalinity produced by 1 gm. of magnesia. 5. The probable error shows that the method is accurate.

THE MECHANISM OF THE DIFFUSION OF ELECTROLYTES THROUGH THE MEMBRANES OF LIVING CELLS.

I. THE NECESSITY OF A GENERAL SALT EFFECT UPON THE MEMBRANE AS A PREREQUISITE FOR THIS DIFFUSION.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 11, 1916.)

INTRODUCTION.

In this series of publications the writer intends to prove the existence of what he believes to be a new principle in the mechanism of the diffusion of certain electrolytes through the membranes of living organs or cells; namely, that this diffusion depends, in addition to the osmotic pressure of the electrolyte, upon a second effect which he calls the general "salt effect" upon the membrane (or certain constituents of the membrane).[1] Last year Loeb and Cattell published the observation[2] that when eggs of *Fundulus* were washed for 24 hours in distilled water and then transferred to a solution of a potassium salt, it took a longer time before the hearts of the embryos inside the egg stopped beating than when the eggs were transferred directly from sea water into the same solution of the potassium salt. This result was ascribed by us to the fact that in the distilled water the eggs lose the salts which are in chemical combination with the surface or the superficial layer of the membrane, and that this lack of salts at the surface was the reason for the retardation of the diffusion of the potassium salts into the egg. This view was supported by the measurements of the osmotic pressure of the juice pressed out from washed and unwashed eggs,[3] the osmotic

[1] Loeb, J., *Proc. Nat. Acad. Sc.*, 1916, ii, 511.

[2] Loeb, *Proc. Nat. Acad. Sc.*, 1915, i, 473. Loeb, J., and Cattell, McK., *J. Biol. Chem.*, 1915, xxiii, 41.

[3] Loeb, J., and Wasteneys, H., *J. Biol. Chem.*, 1915, xxiii, 157.

pressure of the latter being a trifle higher. It is our intention to show in this paper that the diffusion of KCl into washed eggs can be accelerated by adding to the KCl solution a moderate amount of salt; *e.g.*, sea water or a mixture of NaCl and $CaCl_2$ or NaCl alone, thus proving that the retardation of the diffusion of potassium salts into washed eggs is due to the lack of salts at the surface of the egg membrane. The presence of these salts at the external surface of the egg membrane furnishes that second factor—the salt effect—required for the diffusion of the potassium salts. When washed eggs are put into a pure solution of KCl the diffusion of the latter salt into the egg (*i.e.*, through the external surface or layer of the membrane) is delayed until so much KCl has combined with certain elements of the surface of the membrane as to supply the "salt effect."

Before entering into a description of the experiments it will be well to prove that we are dealing in these experiments with an action upon the membrane of the egg and not upon the embryo or upon its heart. It is one of the shortcomings of the usual experiments on salt action that we are at a loss to decide whether we are dealing with a salt action on the membrane or on the adjacent protoplasm. Thus when we find that $CaCl_2$ inhibits the twitching of muscles or nerves caused by Na_2SO_4, Na_3 citrate, or $N(C_2H_5)_4Cl$, we may be in doubt whether this is due to a prevention of the diffusion through the membrane or to an action of Ca upon the living protoplasm, though the evidence speaks in favor of the former possibility.[4] In the experiments on the eggs of *Fundulus* we are relieved of this difficulty since we can always compare the action of the salt on the egg with its action upon the embryo just hatched and thus freed from the protection of the egg membrane. Such a comparison gives us the certainty that in our experiments we are dealing with the effects of salts upon the egg membrane which we must conceive as impermeable for water and nearly impermeable for salts.

We shall see in this paper that an M/8 solution of KCl causes cessation of the heart beat of the embryo in the washed egg within a certain time, that the addition of a definite but small quantity of the salts of sea water or of NaCl and $CaCl_2$ accelerates

[4] Loeb, J., and Ewald, W. F., *J. Biol. Chem.*, 1916, xxv, 377.

the poisoning, and that a slightly higher concentration of the same salts inhibits the poisoning. That all these phenomena are due to an action on the membrane of the egg is proved by the fact that two salts utterly incompatible with the life of the embryo or with heart action, *e.g.*, $NaNO_3$ + $MnCl_2$, will produce the same effect as NaCl + $CaCl_2$. This would be impossible of explanation on any other basis than that NaCl + $CaCl_2$ as well as $NaNO_3$ + $MnCl_2$ produce the salt effect as well as the antagonistic effect by acting on the membrane of the egg and not by acting on the heart or the embryo.

In 1905[5] the writer expressed the idea that the antagonistic action of traces of a salt with bivalent cation to high concentrations of a salt with univalent cation in the egg of *Fundulus* was due to a prevention of the diffusion of the latter salt through the membrane; and this conclusion was based on the following fact. When newly fertilized eggs are put into a 5/8 M NaCl solution no egg can form an embryo, while the addition of a trace of $ZnSO_4$, lead acetate, $MnCl_2$, or practically of any salt with a bivalent cation, allowed all or many of the eggs to form embryos. When, however, the newly hatched fish is put into a 5/8 M NaCl solution it dies even more quickly if a trace of $ZnSO_4$ or lead acetate or $MnCl_2$ is added than in the pure 5/8 M NaCl solution. For the embryo outside the membrane of the egg only Ca or Sr or Mg can be used as antagonists to higher concentrations of NaCl. This shows that the prolongation of the life of the egg in a 5/8 M NaCl solution through the addition of a bivalent cation is due to a modification of the membrane by the latter whereby the membrane is rendered impermeable for these salts.

Moreover when we put the eggs of *Fundulus* with beating hearts into a pure 3 M solution of NaCl the eggs are killed in about 3 hours while they live for 3 to 5 days when we add the proper amount of Ca.[6] We can prove with certainty that the Ca acts by preventing the diffusion of the NaCl into the egg, since if we put the newly hatched embryos into the same 3 M NaCl + $CaCl_2$ solution in which the unhatched embryo of the same age lives 3 to 5 days, they die in less than 10 minutes. The young larvæ of the fish freed from the membrane cannot live

[5] Loeb, *Arch. ges. Physiol.*, 1905, cvii, 252.

[6] Loeb, *Science*, 1912, xxxvi, 637; *Biochem. Z.*, 1912, xlvii, 127.

in a solution of NaCl + $CaCl_2$ higher than 10/8 M.[7] Hence we may be certain that only traces of a solution of 3 M NaCl + $CaCl_2$ diffuse through the membrane of the eggs so that it takes from 3 to 5 days to allow so much NaCl + $CaCl_2$ to diffuse through the membrane as is required to raise the concentration inside the membrane to 10/8 M or above.

This statement can be corroborated by determinations of the specific gravity of fertilized eggs of *Fundulus*. The method of these experiments consisted in ascertaining the concentration of NaCl solution in which the eggs were just able to float. The normal egg of *Fundulus* has a specific gravity higher than sea water but less than that of a 14/8 M NaCl solution.[8] Thus it was ascertained that eggs kept for 6 days in sea water floated in 14/8 M and sometimes in 13/8 M NaCl; eggs of the same female kept 6 days in distilled water floated in 13/8 M NaCl and sometimes in 12/8 M NaCl; eggs kept for 3 days in 3 M NaCl + $CaCl_2$ sank at first in a 15/8 M NaCl solution but rose again in a few minutes and remained at the surface (after having lost the salt solution which adhered at their surface when first brought into the 15/8 M NaCl from the 3 M NaCl + $CaCl_2$ solution). They were not able to float in a 14/8 M NaCl solution. This experiment gives an indication of how slowly a balanced solution or H_2O diffuses into or through the membrane. In a 3 M NaCl + $CaCl_2$ solution the specific gravity of eggs increased in 3 days only about 7 per cent and in H_2O the specific gravity diminished to about the same amount in 6 days.

Since from our experiments we drew conclusions upon the diffusion of KCl through the membrane of the egg it may be well to show the difference of the action of KCl solutions upon the embryo when inside the membrane and when freed from the membrane. The embryo inside the egg can live more than a week in a pure M/16 KCl solution, especially when the egg is previously washed in H_2O or in dilute sea water; *e.g.*, M/80. The fish themselves succumb to a pure M/16 KCl solution in less than 1 hour, no matter whether they are put directly into the M/16 KCl solution from normal (M/2) sea water or whether they are previously kept for 6 days in M/80 sea water. In the

[7] Loeb, *Biochem. Z.*, 1913, liii, 391.

[8] Loeb, *Biochem. Z.*, 1912, xlvii, 127.

newly hatched fish the heart stopped beating in an M/8 KCl solution in considerably less than 30 minutes, often in 10 minutes, while the embryos of the same lot of eggs but still inside the membrane were not poisoned by the same KCl solution in 24 hours, and if the eggs were previously washed in H_2O or M/80 sea water they could live in such a solution as long as 6 days. This proves that H_2O as well as the salts penetrate the membrane extremely slowly and that the salt effect as well as the antagonistic salt action to be described in this paper are due to modifications of the egg membrane and not to an action upon the embryo itself.

In our experiments we generally only modify the salt contents of the external surface or layer of the egg membrane. The internal layer is in contact with the liquid of the egg, *e.g.*, a solution with a freezing point depression of between 0.75 and 0.77°.[3] It is probable that the osmotic pressure of the liquid inside the egg is furnished chiefly by electrolytes. This liquid is generally not affected by the washing of the eggs.

I. The Difference in the Rate of Diffusion of Potassium Salts into Washed and Unwashed Eggs.

Eggs washed for 24 hours in distilled water and unwashed eggs (taken directly from sea water) were put into different concentrations of a potassium salt, *e.g.*, KCl or K_2SO_4, and after definite intervals the rate of poisoning in the two sets of eggs was ascertained by counting the percentage of hearts still beating in each solution. The smaller this percentage at a given time, the greater the rate of poisoning. Each solution contained about twenty eggs (about 1 week old and with heart beat and circulation well established). Tables I and II show the difference in the rate of poisoning between the washed and unwashed eggs. This difference corresponds exactly with our theory. The *unwashed* eggs (Table I) contain at the beginning enough salt at the surface of their membrane to permit the rapid diffusion of K_2SO_4 into the egg. Accordingly we notice that in all solutions a sharp drop in the percentage of beating hearts occurs during the first 2 or 3 hours, during which time the surface of the membrane has still enough salt for the salt effect. After this period the further drop in the percentage of heart

TABLE I.

After	Percentage of unwashed eggs (taken directly from sea water) with beating hearts in K_2SO_4.							
	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256
hrs.								
½	80	85	100	100	100	100	100	100
1	50	75	80	80	75	75	95	100
2	0	30	50	50	65	50	95	95
5		20	45	45	45	50	80	80
11		10	40	40	40	50	65	70
22		10	35	40	55	55	70	70
48		0	15	40	45	50	70	70
78			10	35	40	55	65	70
123			5	25	30	45	60	75
175			0	35	30	20	45	75
267				0	5	0	5	15
343					0	0	0	0

beats is slow, for the reason that by this time the greater amount of salts of the sea water has diffused from the surface of the membrane into the outside solutions. From now on the further diffusion of K_2SO_4 into the egg is retarded until enough of this salt has entered into combination with the outer surface (or layer) of the membrane to supply the general salt effect.

On the other hand, the washed eggs (Table II) contain at the beginning no or only an inadequate amount of salt at the surface of their membrane, and hence according to our theory the diffusion of K_2SO_4 through the membrane should be impossible. A glance at Table II shows that this is the case, inasmuch as during the first 2 hours not a single heart stops beating in the solutions below M/2 K_2SO_4 and during the first 12 hours none stop beating in the solutions below M/8 K_2SO_4. The diffusion of K_2SO_4 into the washed eggs can only begin after enough K_2SO_4 has combined with the outer surface or layer of the membrane to furnish the general salt effect required for the diffusion of the potassium salts through the membrane. This happens the more rapidly the higher the concentration of the K_2SO_4 solutions. After once the surface of the membrane is supplied with the necessary amount of salt for the salt effect, the hearts stop beating rather rapidly.

TABLE II.

After	Percentage of washed eggs with beating hearts; *i. e.*, of eggs kept for 24 hrs. in distilled water before being put into K_2SO_4.							
	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256
hrs.								
1	90	100	100	100	100	100	100	100
2	35	100	100	100	100	100	100	100
4	0	75	100	100	100	100	100	100
12		50	100	100	100	100	100	100
23		5	85	100	100	100	100	100
47		0	80	100	95	85	100	100
78		0	80	100	90	80	95	100
123			40	92	90	50	85	95
177			15	70	50	45	55	75
268			5	5	10	0	5	15
343			0	0	0	0	0	0

It may perhaps be desirable to follow these results a little more in detail. We begin with the M/8 K_2SO_4 solution.

Half of the *unwashed* eggs stop beating in an M/8 K_2SO_4 solution in 2 hours (Table I), while not a single one of the *washed* eggs shows a cessation of the heart beat in the same time in an M/8 K_2SO_4 solution (Table II), and only a slight poisoning effect appears in the washed eggs in an M/8 K_2SO_4 solution until the 4th day. From that time on a more rapid increase in the percentage of poisoned hearts is found in the washed eggs owing to the gradual production of a salt effect upon the membranes by the M/8 K_2SO_4 solution itself. In the *unwashed* eggs there is a rapid drop in the percentage of beating hearts during the first few hours (Table I); after this time the number of eggs with beating hearts remains almost constant, since the salts have diffused away from the surface of the eggs into the outside solutions. From now on the further salt effect upon the membrane has to be supplied by the M/8 KCl solution itself. This is a slow process and we therefore notice a very slow increase in the percentage of poisoned eggs after the first 6 hours in the unwashed eggs (Table I). The same difference exists for the M/16, M/32, and M/64 K_2SO_4 solution. Half of the *unwashed* eggs stop beating in these solutions in the first 3 hours, owing to the presence of the salts of the sea water at the surface

of the membrane; while it requires a week or more before the same effect is produced in the *washed* eggs by the same solutions.

In the M/4 solution, 70 per cent of the unwashed eggs had stopped beating in the first 2 hours (Table I), while not a single one of the washed eggs (Table II) was poisoned in this time, owing to the lack of salts at the surface of the latter and of their presence at the surface of the unwashed eggs. After this time the M/4 K_2SO_4 is beginning to produce the salt effect and now the washed eggs are poisoned almost as rapidly as the unwashed eggs. This is shown also in the fact that it requires about as much time to poison the last egg of the unwashed as of the washed eggs in the M/4 K_2SO_4 and the weaker solutions. In the M/2 K_2SO_4, the difference between the washed and unwashed eggs becomes less marked simply because the M/2 K_2SO_4 solution can produce the salt effect upon the membrane of the washed eggs more rapidly.

Similar observations as in the case of K_2SO_4 were made in regard to the effect of KCl solutions upon washed and unwashed eggs. Thus more than 50 per cent of the hearts of unwashed eggs ceased to beat in an M/8 KCl solution after less than $1\frac{1}{2}$ hours while 50 per cent of the hearts in the washed eggs were still beating after 2 days in the same solution.

These experiments therefore harmonize with the idea that the rapidity of the diffusion of a potassium salt through the membrane of the egg of *Fundulus* depends, in addition to the osmotic pressure of the KCl solution, upon the presence or absence of salt at the surface or the superficial layers of this membrane.

II. The Acceleration of the Diffusion of KCl through the Addition of a Moderate Amount of a Different Salt, and the Retardation of this Diffusion by the Addition of a Greater Amount of Salt.

We can prove the correctness of the last statement by putting washed eggs of *Fundulus* (*a*) into pure M/8 KCl solutions and (*b*) into M/8 KCl solutions to which some sea water or some other salt solution has been added. If it is true that the hearts of the washed eggs in the previous experiments stopped so much later because their membranes did not contain enough salt, the addition of some sea water or some other salt solution should

accelerate the poisoning of washed eggs in an M/8 KCl solution; and the more so the higher (up to a certain limit) the concentration of the sea water. Eggs were put for 24 hours in H_2O (after having been washed three times in H_2O) and were then distributed in M/8 KCl solutions made up in H_2O and in increasing concentrations of sea water up to normal sea water. We call the concentration of our sea water M/2 although it is a trifle higher, and we call twice diluted sea water M/4, and so on. Table III gives the result of the experiment.

TABLE III.

After	Percentage of washed eggs with beating hearts in M/8 KCl made up in sea water or H_2O.								
	Sea water.								H_2O
	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	
days									
1	24	70	46	65	63	72	85	82	96
2	10	20	30	44	55	65	55	63	80
3	35	0	16	34	28	42	55	50	55
4	30	0	8	8	14	36	20	37	45
5	10	0	4	0	9	27	15	25	25
6	0	0	0	0	9	18	15	18	15
7	0	0	0	0	9	13	10	6	0

While it was more than 3 days until 50 per cent of the hearts of the washed eggs stopped beating when put into a pure M/8 KCl solution, in an M/8 KCl solution in undiluted sea water it took considerably less than a day, and in an M/8 KCl solution made up in twice diluted (M/4), four times diluted (M/8), and eight times diluted (M/16) sea water it took less than 2 days. In M/8 KCl dissolved in M/128 and M/256 sea water the eggs behaved as they did in the pure M/8 KCl solutions. This proves that our contention was correct that the difference between the rate at which washed and unwashed eggs were poisoned by the solution of potassium salt was due to lack of salts on the surface (or the superficial layer) of the membrane. Another interesting fact appears in these experiments. M/4 sea water, while less efficient during the first 2 days of the experiment than undiluted sea water, becomes more efficient from the 3rd day on;

because, as we assume, the amount of salts which diffuses into the surface layer of the membranes in the undiluted sea water exceeds the quantity required for the acceleration of the diffusion of KCl and the opposite effect is produced; namely, a retardation (antagonistic salt action).

Since the sea water contains a small amount of KCl it was necessary to show that the substitution of a physiologically balanced salt solution (free from KCl) for sea water gives the same result. Such a solution affords us also an opportunity to prove *that while the addition of a moderate amount of salt* (*e.g.*, M/8 *or* M/4) *accelerates the rate of diffusion of* M/8 *KCl into the washed eggs, a slightly higher concentration of* $NaCl + CaCl_2$, M/2 *or* M/1, *has the opposite effect; namely, to retard or prevent the diffusion of KCl into the egg*, with the exception of the first hour or more, when they also accelerate the diffusion.

Eggs washed for 24 hours in H_2O were distributed into M/8 KCl solutions made up in H_2O and in different concentrations of NaCl + $CaCl_2$ (in the proportion in which these salts are contained in sea water, 100 molecules of NaCl to 1.75 molecules of $CaCl_2$).

TABLE IV.

After	Percentage of washed eggs with beating hearts in M/8 KCl in NaCl + $CaCl_2$ or H_2O.									
	NaCl + $CaCl_2$									H_2O
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	
days										
1	100	73	74	90	95	96	87	75	85	96
2	100	77	38	64	85	84	66	40	85	80
3	100	77	9	20	40	25	53	20	85	55
4	100	73	0	0	25	10	50	20	80	45
5	100	67	0	0	20	10	47	20	70	25

We notice the same fact as in the experiment with sea water; namely, that if an M/8 KCl solution is made up in M/4 or M/8 solution of NaCl + $CaCl_2$ the velocity with which KCl diffuses into the egg is accelerated, thus proving that the action of sea water was not due to the KCl it contained. Thus after 3 days, more than 50 per cent of the eggs had still beating hearts in the

pure M/8 KCl solutions, while in the M/8 KCl solution made up in M/4 NaCl + $CaCl_2$ only 9 per cent had beating hearts. After 3 days all the hearts had stopped beating in M/8 KCl solutions made up in M/4 and M/8 NaCl + $CaCl_2$ while in the pure M/8 KCl solutions still 45 per cent of the eggs had beating hearts.

The M/8 KCl solution made up in M/2 NaCl + $CaCl_2$ is of special interest. During the 1st day (and very probably during the first hours of the 1st day) the number of eggs with beating hearts fell to 73 per cent while in the pure M/8 KCl solution it fell only to 96 per cent. Therefore the M/2 NaCl + $CaCl_2$ at first accelerates the diffusion of KCl through the membrane. This is quite intelligible on the basis of our theory. The equilibrium between the surface of the membrane and the salt solution was not established at once but only gradually, and before the equilibrium was complete there existed a condition in which the membrane had as much salt as corresponded to the equilibrium with an M/8 and later a M/4 solution of NaCl + $CaCl_2$ which accelerate the diffusion. During this period 27 per cent of the eggs were poisoned. Then more salt entered into combination with the membrane and the opposite result was produced; namely, an impermeability to M/8 KCl. We therefore see that during the next 4 days no more eggs were poisoned with KCl. In the M/1 NaCl + $CaCl_2$ this latter condition was produced so rapidly that there was no chance for the diffusion of enough KCl into the eggs to cause the cessation of heart beats. All the hearts were still beating in one experiment in an M/8 KCl solution made up in M/1 NaCl + $CaCl_2$ + $MgCl_2$ + $MgSO_4$ after 19 days, when the experiment was discontinued.

The preceding experiments have shown that the acceleration of the rate of poisoning with M/8 KCl in M/8 or M/4 sea water is due to a salt action upon the membrane. We can also show that the prevention of the poisoning by an M/8 KCl solution through a M/1 or M/2 solution of NaCl + $CaCl_2$ is not due to an action of these latter salts upon the heart, but to an action upon the membrane. This proof is furnished by the fact already mentioned in the introduction, that a solution utterly incompatible with the activity of the heart of *Fundulus*, *e.g.*, $NaNO_3$ + $MnCl_2$, acts in the same way as NaCl + $CaCl_2$, as shown in Table V. Eggs washed 24 hours in H_2O were put into M/8 KCl

made up in H_2O and different concentrations of $NaNO_3 + MnCl_2$ (100 molecules of the $NaNO_3$ to 1.75 molecules of the $MnCl_2$).

TABLE V.

After	Percentage of washed eggs with beating hearts in M/8 KCl in $NaNO_3 + MnCl_2$ or H_2O.							
	$NaNO_3 + MnCl_2$							H_2O
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	
days								
1	100	45	44	44	90	85	90	85
2	100	45	40	58	85	75	75	60
3	100	45	30	50	75	60	50	50
4	90	45	10	20	45	65	50	40
6	70	34	0	0	33	30	30	10

This experiment shows that the M/8 KCl solution was not able to poison any of the eggs when the solution was made up in a M/1 solution of $NaNO_3 + MnCl_2$. This protective or antagonistic action of $NaNO_3 + MnCl_2$ could only have been due to a salt effect upon the membrane, whereby the diffusion was prevented. While in the pure M/8 KCl solution 85 per cent of the embryos were still alive after 1 day, in the M/8 KCl solution made up in M/2, M/4, and M/8 solution of $NaNO_3 + MnCl_2$ more than half of the hearts of the embryos had stopped beating in the same time; while in the M/8 KCl made up in M/16, M/32, and M/64 solution of $NaNO_3 + MnCl_2$ the embryos behaved like those in the pure M/8 KCl. We notice again the characteristic phenomenon that in M/8 KCl made up in M/2 $NaNO_3 + MnCl_2$ during the 1st day a sharp drop occurs in the number of beating hearts (to 45 per cent), while in the pure M/8 KCl solution at this time only 15 per cent of the hearts have stopped beating. After that the number of eggs with beating hearts remains constant in the M/8 KCl solution made up in M/2 $NaNO_3 + MnCl_2$ while in the pure M/8 KCl solution it diminishes constantly. The explanation is the same as given for $NaCl + CaCl_2$.

Since it might be thought that this antagonistic action was due to the presence of the bivalent cation Ca or Mn in the solution we may state that non-balanced salt solutions act in the same way. The following experiment (Table VI) with NaBr may

serve as an example. Eggs washed for 24 hours in H_2O were put into M/8 KCl made up in different concentrations of NaBr.

TABLE VI.

After	Percentage of washed eggs with beating hearts in M/8 KCl in NaBr, H_2O, or sea water.										
	NaBr									H_2O	M/4 Sea water.
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256		
days											
1	100	95	52	47	75	65	70	74	85	95	35
2	92	85	40	40	65	55	60	57	80	75	15

It is obvious that M/1 and M/2 NaBr solutions prevented the diffusion of KCl into the eggs since practically the hearts of all of them continued to beat. In the M/8 KCl solution made up in M/4 and M/8 NaBr the eggs were poisoned more rapidly than in the pure M/8 KCl solution and the same was true for the eggs in the M/8 KCl solutions made up in M/4 sea water. The M/8 KCl solutions made up in M/16 to M/256 NaBr acted almost like the pure M/8 KCl solution.

CONCLUSION.

All these experiments prove that the diffusion of KCl through the membrane of the egg of Fundulus does not depend only on the osmotic pressure of the KCl solution but that in addition a certain modification of the membrane is required (*general salt effect.*) *This modification is produced by the action of salts upon the external surface or layer of the membrane* (presumably upon the proteins of the membrane). When the eggs are in the sea water the salts of the latter supply this general salt action upon the membrane. When the membranes are sufficiently freed from the salts of the sea water by washing them for 24 hours in H_2O and if they are then put into a pure solution of a potassium salt the poisonous effect of the potassium salt is delayed until the potassium salt itself has had time to supply the salt effect upon the membrane required to permit the diffusion of this salt through the membrane. When the concentration of the potassium salt is low its diffusion into the washed eggs of *Fundulus* can be accelerated by adding some other balanced or non-balanced salt solution, which supplies the salt effect upon the membrane.

When the concentration of the salt solution added to the KCl solution is slightly higher than that required for the accelerating salt effect upon the membrane, the opposite phenomenon is observed; namely, the retardation or prevention of the diffusion of the potassium salt. This latter effect, the antagonistic salt action, has thus far alone aroused the attention of the workers, although the general salt effect discussed in this paper was already noticed 6 years ago when we found that the adult fish of *Fundulus* die more rapidly in M/100 to M/66 KCl solutions when 10 molecules of NaCl are added to 1 molecule of KCl, while the opposite effect, namely, an antagonistic action, results when more (17 molecules or more) NaCl is added to the 1 molecule of KCl.[9] The meaning of the fact, *i.e.*, the necessity of a general salt effect for the diffusion of potassium, was at that time not recognized, but we can now explain these observations on the assumption that while the gills or skin of the fish contain enough salt to permit the diffusion of M/100 KCl through the surface a better salt effect is reached when about 10 molecules of NaCl are added to 1 molecule of KCl. The addition of 17 or more molecules of NaCl to 1 molecule of KCl has the opposite effect of retarding the diffusion of KCl (antagonistic salt action). This observation shows that the phenomena described for the membrane of the egg of *Fundulus* are not confined to this object but are more general in character.

It has become customary in physiology to discriminate between diffusion and secretion, the term diffusion being used when the difference in pressure of the diffusing substance on both sides of a membrane is sufficient to account for the direction of the diffusion; while we speak of secretion when this is no longer possible. Some authors imply that in the latter case specifically vital conditions or conditions inseparable from the life of the cell play a rôle. The question whether the exchange of gases in the lungs is a mere process of diffusion or one of secretion has divided physiologists for some time. It is quite possible that the facts published in this paper will help towards lifting the veil of mystery from certain cases of secretion.

[9] Loeb and Wasteneys, *Biochem. Z.*, 1911, xxxii, 155.

THE MECHANISM OF THE DIFFUSION OF ELECTROLYTES THROUGH THE MEMBRANES OF LIVING CELLS.

II. THE DIFFUSION OF KCl OUT OF THE EGG OF FUNDULUS AND THE RELATIVE EFFICIENCY OF DIFFERENT IONS FOR THE SALT EFFECT.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 15, 1916.)

I. The Impossibility of Recovery from Potassium Poisoning in Solutions of Non-Electrolytes.

We showed in the first part of this series[1] that the diffusion of potassium salts through the membrane of the living egg of *Fundulus* depends, in addition to the osmotic pressure of the solution, on a second effect which we called the general salt effect. Thus if the external surface of the membranes of the eggs is freed from salt by washing for 24 hours in distilled water, the potassium salts cannot diffuse into the egg until the external surface of its membrane has been again supplied with salts either by adding a second salt or by giving the potassium salt in solution time to enter into combination with certain constituents of the membrane and thus to supply the general salt effect.

Loeb and Cattell[2] have already shown that by depriving the external surface of the membrane of salts an obstacle is created also for the diffusion of potassium salts in the opposite direction; namely, from the interior of the egg into the surrounding solution. When eggs of *Fundulus* are poisoned with KCl until all the hearts stop beating, they can recover and the hearts can begin to beat again, as soon as enough KCl has diffused out of the egg to

[1] Loeb, J., *J. Biol. Chem.*, 1916, xxvii, 339.

[2] Loeb, J., and Cattell, McK., *J. Biol. Chem.*, 1915, xxiii, 41.

bring the concentration of KCl inside the egg below that limit which is required for the cessation of the heart beat. Loeb and Cattell found that eggs kept in a KCl solution until all the hearts stopped beating could not recover when put subsequently into distilled water or a solution of cane sugar, while they could recover when put into solutions of electrolytes. The same authors have also shown that in this recovery from potassium poisoning the nature of the anion in the outside solution played an important rôle, inasmuch as the relative efficiency of Cl : SO_4 : citrate was approximately as 1 : 4 : 16 (Hardy's rule).

In our new experiments the eggs were put directly from sea water into a M/2 KCl solution where they remained for 12 hours. Since the hearts stopped beating, as a rule, in less than 2 hours in such a solution, each egg contained an amount of KCl considerably in excess of that needed for the cessation of the heart beat. Each egg also probably contained approximately the same amount of KCl. When such eggs are put from the M/2 KCl into distilled water, they contain at first enough salt at their surface to permit the diffusion of KCl through the membrane, and we may therefore expect that in the first few hours a few of the eggs may recover even in distilled water. But after a few hours the external surface will have lost a considerable part of its salts by diffusion into the outside solution and as a consequence this external surface or layer of the membrane will become impermeable for the KCl and from then on no more eggs should be able to recover. This was actually observed for eggs which were put into H_2O after treatment with KCl and we will show that the same is true if eggs poisoned with KCl are put into any solution of a non-electrolyte.

We first give as an illustration the behavior of eggs poisoned for 12 hours in M/2 KCl and then put into glycerol solutions of different concentrations (Table I). By recovered eggs we mean eggs whose hearts beat regularly again. While 75 per cent of the eggs recovered in sea water inside of 6 hours and all recovered in 24 hours, of those put into H_2O or glycerol solutions very few recovered. The important fact is that the small number of recoveries observed in glycerol and H_2O took place during the first 6 hours only, while the external surface of the egg membrane had not yet lost that quantity of salts required for the general

TABLE I.

After	Percentage of poisoned eggs which recovered in glycerol solutions, sea water, or H_2O.										
	Glycerol.									M/2 Sea water.	H_2O
	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512		
days											
¼	26	10	10	11	11	0	10	0	15	75	5
1	26	20	15	11	11	0	15	5	15	100	10
2	21	10	15	11	5	0	10	5	20		10
3	10	10	20	11	5	0	5	5	10		5
4	21	10	10	5	5	0	5	5	10		5
5	21	10	5	5	5	0	5	0	5		5
Eggs then transferred to sea water.											
1	89	100	95	100	100	100	95	95	100		

salt effect upon the membrane. After this no further recovery took place in the next 5 days. Then the eggs were transferred into sea water where the salt effect was supplied. In 24 hours practically all the eggs had recovered; the figures are given above.

Table II gives a similar experiment with cane sugar.

TABLE II.

After	Percentage of eggs which recovered in cane sugar or H_2O.											
	Cane sugar.											H_2O
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	
days												
¼	14	5	14	5	9	5	15	38	11	23	5	0
1	14	10	14	20	14	16	15	38	22	34	10	0
2	14	15	14	15	5	10	15	22	11	34	10	0
3	14	10	9	10	14	10	5	22	11	23	0	0
4	9	10	9	10	14	5	5	22	0	34	0	0
5	9	10	14	10	5	0	0	0	0	29	0	

Again we notice the same striking phenomenon that what little recovery there is in the solutions of cane sugar takes place during the first 6 hours when the salt has not yet diffused in sufficient quantities from the external surface of the membrane. After 5 days the number of recoveries has not increased beyond that

which took place during the first 6 hours. The number of recoveries which took place during the first 6 hours was comparatively large, owing to the fact that either the salt content of these eggs was slightly greater than in the glycerol experiment, or the salts diffused away from the surface of the membrane with less rapidity than in the case of other non-electrolytes.

TABLE III.

After	Percentage of eggs which recovered in grape sugar or H_2O.										
	Grape sugar.										H_2O
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	
days											
$\frac{1}{4}$	5	15	15	5	0	0	5	5	5	0	10
1	0	15	10	5	0	0	0	5	0	0	15
2	10	15	20	5	0	5	0	5	0	0	10
The eggs were then transferred to sea water. The recovery took place rather rapidly.											
1	74	91	85	75	85	75	66	60	65	70	65
2	100	95	95	100	100	95	95	85	85	90	85

Finally we give a third experiment on the recovery of poisoned eggs in grape sugar (Table III). As in the previous experiments with non-electrolytes a small percentage of recoveries occurred in grape sugar during the first 6 hours when not enough salt had diffused away from the surface of the membrane. After the first hours no further recoveries took place because the outer layer and surface did not contain enough salt for this purpose. As soon, however, as the eggs were put into sea water the recovery became very rapid since then the outer surface and layer of the membrane was supplied with salt.

Experiments with other non-electrolytes (or very weak electrolytes) like ethyl and methyl alcohol and urea gave about the same results as those reported. From all these experiments we can draw the following conclusion: *The diffusion of KCl out of the interior of the poisoned Fundulus eggs cannot take place when the external surface or layer of the membrane contains less salt than that required to produce the salt effect upon the membrane.*

II. The Recovery of Eggs in Balanced and Non-Balanced Solutions.

When we put eggs which had been kept for 12 hours in a M/2 KCl solution into a solution of certain electrolytes instead of non-electrolytes, we observe an altogether different phenomenon from that described for solutions of non-electrolytes. An almost complete recovery of all the eggs takes place in the salt solutions above a certain concentration inside of 6 hours and a gradual recovery in the weaker solutions; since in the weaker solutions enough salt must gradually combine with the outer surface of the membrane to produce enough of a salt effect to allow at least the *slow* diffusion of KCl through the membrane. Table IV gives the recovery in different concentrations of sea water of eggs poisoned for 12 hours previously in M/2 KCl.

TABLE IV.

After	Percentage of eggs which recovered in sea water.											
	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096
days												
¼	75	55	20	10	5	20	5	20	0	10	10	20
1	100	100	95	60	50	26	16	45	14	10	25	35
2			100	80	75	33	33	45	14	10	25	30
3				100	90	70	40	45	14	14	10	20
4					100	100	70	75	22	14	10	10
5							84	100	55	14	10	10
6							95	95	65	20	20	15
7							100	100	72	30	15	15

This experiment was carried on with the same material and simultaneously with the glycerol experiment (Table I). If we compare the two we notice the salt effect; namely, the quick recovery of a majority of the eggs in M/2 and M/4 solutions of sea water within 6 hours. In the previous experiment on the diffusion of the KCl in the opposite direction, *into* the egg, we noticed also that the M/2 and the M/4 sea water accelerated the diffusion of KCl through the membrane in the first 6 hours. No such acceleration is found in the experiments with the non-electrolytes. Moreover, with each day more and more eggs recover in the weaker concentrations of sea water, while no such influence of the concentration can be found in the case of non-electrolytes.

We shall now show that there is no great difference between

the action of a balanced and a non-balanced solution upon the recovery of the heart from KCl poisoning. We compare the percentage of recoveries in a balanced solution, NaCl + $CaCl_2$, and a non-balanced solution, NaCl. Both experiments were made simultaneously. The eggs had been kept for 12 hours in M/2 KCl, as usual.

TABLE V.

After	Percentage of eggs recovering from potassium poisoning in NaCl + $CaCl_2$ or H_2O.										
	NaCl + $CaCl_2$										H_2O
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	
days											
¼	70	85	75	35	5	15	15	15	15	0	20
1	100	95	100	90	55	55	25	25	5	5	20
2		100	100	100	80	75	40	20	10	15	10
3					90	95	60	50	15	5	10
	NaCl										
¼	55	75	80	60	55	45	20	25	10	10	0
1	100	100	100	100	100	90	40	25	15	10	5
2						95	75	35	15	5	5
3						95	80	60	15	10	5

In both solutions more than 50 per cent of the eggs recover in the concentrations above M/4 in less than 6 hours. The pure NaCl solution is more efficient than the mixture of NaCl + $CaCl_2$. In the M/8 and M/16 NaCl solution 60 per cent and 55 per cent of the eggs recover in the first 6 hours while in the M/8 and M/16 solutions of NaCl + $CaCl_2$ only 35 per cent and 5 per cent recover. After 2 days 75 per cent of the hearts have recovered in the M/64 NaCl and only 40 per cent in the M/64 NaCl + $CaCl_2$. This difference is not accidental but constant. The difference is, however, in harmony with our experience with the antagonism between NaCl and $CaCl_2$ if we assume that the $CaCl_2$ makes the NaCl less effective in its action on the membrane and hence less available for the general salt effect; so that a mixture of NaCl + $CaCl_2$ acts like a pure NaCl solution of a lower concentration.

We may consider it as proved that aside from this latter con-

dition balanced solutions like sea water are just as fit to render the diffusion of KCl out of the egg possible as non-balanced solutions, and that it is therefore impossible to ascribe this result to an "injury" to the membrane.

It was of interest to find out whether higher concentrations of NaCl solution or of NaCl + $CaCl_2$ would prevent the diffusion of KCl out of the egg (antagonistic salt action), but this was not the case. Eggs that had been poisoned in M/2 KCl recovered more promptly when put into 3 M, $2\frac{1}{2}$ M, 2 M, and $1\frac{1}{2}$ M NaCl + $CaCl_2$ than when put into M/2, M/4, or M/8 solutions. The probable explanation is that the diffusion of KCl out of the egg took place in these highly concentrated solutions so rapidly that the excess of KCl had already diffused out of the egg before the antagonistic effect of NaCl + $CaCl_2$ could be established. It requires some time until enough molecules of NaCl + $CaCl_2$ combine with the membrane to inhibit the diffusion of KCl. Until this happens the conditions for the diffusion of KCl out of the egg are most favorable so that the eggs recover before the antagonistic salt effect can make itself felt. We shall see in the third part of this paper that if the eggs are first treated for 24 hours with 2 M NaCl + $CaCl_2$ and are then put into a pure M/8 KCl solution, the outer surface of the membrane contains enough NaCl and $CaCl_2$ for the antagonistic salt action and no KCl can diffuse into the egg. The fact that the addition of sea water makes the recovery of the heart possible is in full harmony with the experiments of the first part, that sea water also accelerates the diffusion of low concentrations of KCl into the egg.

The recovery of the egg from the poisoning in the sea water cannot be ascribed to the diffusion of sea water into the egg (which does not occur) since solutions utterly unable to arouse or maintain the heart beat or the life of the embryo facilitate the recovery of the poisoned embryo; *e.g.*, ammonium nitrate and many others.

III. The Influence of Anions and Cations of Different Salts upon the Recovery of Eggs from KCl.

In Table VI we give the percentage of hearts which recovered from KCl poisoning in different solutions of electrolytes. The eggs had previously been 12 hours in a M/2 KCl solution and were then put for 24 hours into the solutions indicated in Table VI.

TABLE VI.

	Percentage of hearts recovering from potassium poisoning in 24 hrs.													
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096	H_2O
Sea water		100	100	91	60	50	26	15	45	15	10	25	35	5
NaCl + $CaCl_2$	100	95	100	90	55	55	25	25	5	5				10
NaCl	100	100	100	100	100	90	40	25	15	10				5
NaBr	100	100	100	100	90	75	66	50	35	30	40			5
$NaNO_3$				100	100	70	35	15	10	15	10	0	10	5
Na_2 acetate		100	100	100	100	100	85	35	15	0	5	5	0	5
Na_2SO_4		100	100	100	100	90	93	90	81	38	14	5		
$NaHCO_3$				100	100	100	100	75	35	10	5			
Na_2CO_3									35	30	20	20	5	5
Na_2HPO_4					95	100	90	55	50	25	10	14	5	0
Na_3 citrate									95	60	80	30	20	
NH_4Cl		38	72	89	83	77	41	16	15	15				11
$(NH_4)_2SO_4$			45	75	70	75	65	65	50	31	15	10	30	10
$(NH_4)_3$ citrate									80	68	55	25	14	
$MgCl_2$			100	85	95	75	55	25	25	25	15	10	0	5
$MgSO_4$		100	95	70	95	90	80	45	70	30	10	10	15	

The order of rising efficiency for the anions for producing the salt effect is as follows:

Cl, NO_3 < Br < Acetate < PO_4 (?) CO_3 < SO_4 < Citrate.

In order to ascertain the influence of valency we may select as a standard of comparison that concentration which causes about 70 per cent of eggs to recover in 24 hours. This concentration is, in the case of Na salts for acetate and approximately also for Br, M/64, for Cl and NO_3 between M/32 and M/64. For SO_4 it is M/256 and for CO_3 between M/128 and M/256. For citrate it is M/1,024 but for PO_4 it is too small, between M/64 and M/128. Omitting Na_2HPO_4 from consideration we find the ratio of efficiency for monovalent: bivalent: trivalent anions as 1 : 4 : 16 which is the expression of Hardy's rule for the influence of valency upon coagulation. It is worthy of notice that the same rule seems to hold for the NH_4 salts. If we compare again the minimal concentrations which allow about 70 per cent

of the eggs to recover, we find that the values are M/32 for NH_4Cl, M/128 for $(NH_4)_2SO_4$ and M/512 for $(NH_4)_3$ citrate which is again $Cl : SO_4$: citrate = 1 : 4 : 16. The values are practically identical with those found for the Na salts, thus showing that we are not dealing with an action of the salts upon the heart since the NH_4 salts are unable to maintain or resuscitate the heart beat.

Table VII gives the influence of the cations upon the recovery.

TABLE VII.

	Percentage of embryos poisoned by KCl recovered after 24 hrs.													
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096	H_2O
LiCl		30	45	80	60	80	60	30	5	0	10			5
NaCl	90	100	100	100	100	90	40	25	15	10				0
RbCl		0	0	0	5	5	0	1	0	2	0			0
CsCl		5	5	0	0	5	5	0	0	0	0			
$MgCl_2$			100	85	95	75	55	25	25	25	15	10		5
$CaCl_2$				100	100	75	45	20	20					
$SrCl_2$				30	15	30	15	20	0	5	25	0	0	10
$BaCl_2$			5	0	5	10	15	25	10	20	35	10	20	0
NH_4Cl		38	72	89	83	77	41	16	15	15				11
$N(C_2H_5)_4Cl$					100	78	60	23	20	14	5	14	12	10

In this case a peculiar influence of the periodic law is noticeable. Li and Na as well as the corresponding members of the next group Mg and Ca favor the recovery, Rb and Cs as well as the corresponding members Sr and Ba inhibit it. NH_4Cl and $N(C_2H_5)_4Cl$ act like NaCl. It is strange that Mg and Ca should increase the permeability in the recovery experiments and have the opposite effect in the case of the diffusion of KCl into the egg. The fact that salts which cannot reestablish the heart beat or maintain it like $N(C_2H_5)_4Cl$ should have almost as powerful an action upon the recovery of the hearts as Na shows that we are dealing with an effect of the salts upon the membrane and not upon the protoplasm of the embryo.

CONCLUSION.

These experiments confirm the conclusion that for the diffusion of the potassium salts through the membrane, aside from the osmotic pressure of the solution, a second factor is required, the general salt effect upon the membrane. While this was proved, in the first part of this series, for the diffusion of potassium salts *into* the egg, it is proved in this part for the diffusion in the opposite direction. It is shown that eggs previously poisoned with KCl cannot recover when put into the solution of any non-electrolyte. A few eggs may recover in such a solution at the beginning, due to the fact that at the beginning the external surface of the egg may have enough KCl for the salt effect upon the membrane. As soon as this salt has diffused away from the surface, no further recovery is possible, since the external surface of the membrane when sufficiently freed from salts is as efficient a barrier for the diffusion of KCl out of the egg as in the opposite direction.

The relative efficiency of different salts for the salt effect upon the membrane is a function both of anion and cation. The efficiency increases with the valency of the anion approximately according to Hardy's rule, Cl : SO_4 : citrate = 1 : 4 : 16, as we had stated already in previous notes; but the efficiency also depends upon the nature of the anion in the following way:

$$Cl,\ NO_3 < Br < Acetate < PO_4,\ CO_3 < SO_4 < Citrate.$$

In regard to the cation we found that Rb and Cs completely inhibit the diffusion of KCl out of the egg in M/2 solution. The salts of Sr and Ba have a similar effect. The salts of Na, Li, Mg, Ca, NH_4, and $N(C_2H_5)_4$ favor the diffusion. Since the salts of Li, NH_4, and $N(C_2H_5)_4$ are extremely toxic for the embryo the beneficial influence of these salts upon the recovery from potassium poisoning must be ascribed to an action upon the membrane and not to an action upon the heart of the embryo. Since the balanced solutions are almost as efficient as non-balanced solutions, the influence cannot consist in a destructive action upon the membrane, but must consist in the creation of special conditions required for the diffusion of electrolytes.

THE MECHANISM OF THE DIFFUSION OF ELECTROLYTES THROUGH THE MEMBRANES OF LIVING CELLS.

III. THE ANALOGY OF THE MECHANISM OF THE DIFFUSION FOR ACIDS AND POTASSIUM SALTS.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 22, 1916.)

I.

We shall show in this paper that the ideas concerning the rôle of the general salt effect developed in the two previous notes[1] hold also for the diffusion of acids into the egg of *Fundulus*. The effect of acids upon the heart of *Fundulus* is not to the same degree reversible as the effect of potassium salts, since when acid diffuses into the egg it first kills and causes coagulation of the body of the embryo from the tail upward and only later causes the heart to stop beating by diffusion through the pericardium. It would be difficult if not impossible to demonstrate the influence of salts upon the recovery of the heart from acid poisoning. We must, therefore, make use of the diffusion of acids *into* the egg for the demonstration of the salt effect in the case of acids. We shall show that eggs washed in H_2O are more resistant to acid than eggs containing some salt. For this demonstration the eggs were first put for 4 days into salt solutions of a different concentration and then exposed to the acid solution. Eggs similarly treated were also exposed to M/8 KCl solutions with this difference only, that it was found sufficient to expose the eggs for 1 day only, previous to their transfer to M/8 KCl. Since the experiments with KCl were more numerous than those with acid, we shall describe them first.

[1] Loeb, J., *J. Biol. Chem.*, 1916, xxvii, 339, 353.

The method employed in the case of the potassium experiments was as follows: The eggs were put for 24 hours into solutions of certain substances varying in concentration. A chemical equilibrium is established between the constituents of the solution and the external surface or layer of the membrane. The inner layer of the membrane is not at all or only little affected. These eggs are then put into an M/8 KCl solution. The hearts will stop beating and the rate at which this happens will give us the rate at which the KCl diffuses. The smaller the percentage of the hearts beating at a certain time, the greater the rate at which the KCl diffuses into the eggs.

For the understanding of these results we must remember that when the egg is transferred from a salt solution into a pure M/8 KCl solution the salts other than KCl will diffuse from the external surface or layer of the membrane into the KCl solution and the greater part of this diffusion will take place in the first hours. We have shown in the first paper of this series that when two lots of eggs are put for 24 hours, one into sea water and the other into H_2O, and then both are transferred to M/8 KCl the main difference in the rate of diffusion of KCl through the membranes of the two lots of eggs will be found in the first 6 hours, since during this period the eggs from the sea water will still have enough salt at the surface of their membranes to supply the salt effect required for the diffusion of KCl through the membrane. Hence more of the eggs taken from sea water will be poisoned during the first 6 hours than of the eggs taken from H_2O; while *after* the first 6 hours the further rate of poisoning will be more nearly the same for the two groups of eggs.

Eggs were put for 24 hours into various concentrations of sea water and then exposed to an M/8 KCl solution. Table I gives the percentage of embryos whose hearts were beating after different intervals in the KCl solution.

The result is quite striking. The eggs taken from normal sea water had in half of the cases enough salt at the surface of their membranes to allow so much KCl to diffuse into the eggs inside of 3 hours that the hearts stopped beating. Of the eggs taken from M/4 sea water only 15 per cent had enough salt at the surface of their membranes to allow the same result. The eggs taken from M/8 or still more diluted sea water behaved like the

TABLE I.

After	Percentage of embryos with beating hearts in M/8 KCl taken from sea water or H_2O.								
	Sea water.								H_2O
	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	
hrs.									
3	55	85	100	100	100	100	100	100	100
5½	50	85	100	100	100	100	100	100	100
8	45	85	100	100	100	100	100	100	100
25	50	85	95	95	100	95	100	100	100

eggs that had been in distilled water, inasmuch as they had not enough salt left at their external surface to permit the diffusion of enough KCl into the egg to cause cessation of heart beat in a single egg (Table I).

It seemed advisable to see what would happen if the experiments were made with eggs kept in balanced solutions of higher concentrations than that of sea water (M/2). Eggs were put for 2 days into mixtures of NaCl + $CaCl_2$ in the proportion of 100 molecules of NaCl to 1.75 molecules of $CaCl_2$ (as in sea water) varying in concentration from 3 M to M/128. From these solutions the eggs were transferred into an M/8 solution of KCl and the percentage of eggs with beating hearts was ascertained after certain intervals (Table II).

This experiment shows that eggs which had been in M/32 NaCl + $CaCl_2$ or in lower concentrations contained so little salt at their surface that the KCl could not diffuse any more rapidly

TABLE II.

After	Percentage of eggs with beating hearts in M/8 KCl taken from NaCl + $CaCl_2$.											
	3 M	2½M	2 M	1½M	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128
hrs.												
3	90	65	0	0	0	0	0	30	80	100	100	100
5½	40	15	0	0	0	0	0	25	75	100	100	100
8	5	5						25	75	100	100	100
25	0	0	0	0	0	0	0	30	75	90	95	95
75	0	0	0	0	0	0	0	15	55	85	80	95

into the egg than if the eggs had been kept in H_2O. The eggs taken from M/16 NaCl + $CaCl_2$ had at the beginning enough salt at their surface so that 20 per cent of the eggs were poisoned in the first 3 hours. In the meantime the diffusion of the NaCl + $CaCl_2$ from the surface of the membranes took place so rapidly that afterwards no more eggs were poisoned in the next 25 hours and only a few more in the following 2 days. The eggs taken from the M/8 solution of NaCl + $CaCl_2$ had enough salt at their surfaces to allow the diffusion of a poisonous dose of KCl into 70 per cent of the eggs in the first 3 hours, and in the meantime so much of the $CaCl_2$ + NaCl left the surface of the membrane that the eggs from then on behaved like washed eggs, so that in the next 25 hours the number of eggs poisoned did not increase, and increased only slightly in the next 2 days. All the eggs that had been in the next higher solutions, from M/4 to 2 M, were completely poisoned by M/8 KCl in less than 3 hours, which means that they had enough salt at the surfaces of their membranes to allow the diffusion of a poisonous dose of potassium through the membrane in less than 3 hours. The eggs, however, that had been in $2\frac{1}{2}$ and 3 M NaCl + $CaCl_2$ show another picture; they had an excessive amount of salt at the external surface of their membranes which made them for a number of hours immune against the M/8 KCl solution (antagonistic salt action). This immunity did not last long, however, since the Na and Ca diffused from the surface of the membrane into the M/8 KCl solution and then after about 5 hours a stage was reached when the eggs had only that moderate amount of salt at the external surface of their membrane which accelerates the diffusion of a toxic dose of KCl into the egg. This happens more quickly in the case of the eggs that had been kept for 2 days in a 5/2 M than in a 3 M solution. The same fact, that a moderate amount of salt at the surface of the egg is required to permit the diffusion of KCl into the egg while a higher concentration prevents the diffusion, is shown in the following experiment. Eggs had been put for 2 days into a mixture of NaBr + $CaCl_2$ (in the same proportion as in sea water) and were then transferred into M/8 KCl. Table III gives the percentage of eggs whose hearts stopped beating after several hours.

Again we see that a previous treatment with a moderate concentration (M/8 to M) of a balanced solution accelerates the rate of diffusion of KCl into the egg while previous treatment with a more concentrated solution retards it. But this retardation lasts only through the first hours, as long as the NaBr has not diffused away from the surface of the membrane. It is noteworthy that the inhibiting or antagonistic concentration is lower for NaBr + $CaCl_2$ than for NaCl + $CaCl_2$, suggesting possibly a firmer attachment to the membrane in the case of NaBr than in the case of NaCl. It is also obvious that the general salt effect of sea water is less than that of NaCl + $CaCl_2$ or of NaBr + $CaCl_2$ solutions of equal concentration. This difference must be due to the presence of some of the other constituents in sea water than NaCl and $CaCl_2$.

TABLE III.

After	Percentage of eggs with beating hearts in M/8 KCl after having been kept for 48 hrs. in NaBr + $CaCl_2$ or H_2O.										
	NaBr+$CaCl_2$										H_2O
	2 M	1½ M	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	
hrs.											
3	81	15	0	0	0	15	70	100	100	100	100
5½	60	10	0	0	0	15	65	95	100	100	
8	20	5	0	0	0	10	65	95	100	100	
25	0	0	0	0	0	10	60	90	100	100	
75	0	0	0	0	0	5	45	70	85	80	

Considering the theoretical importance of these experiments, one more observation of the same type may be recorded, in which the eggs had been put for 17 hours into various concentrations of a Ringer solution (NaCl + KCl + $CaCl_2$) and for controls in M/2 sea water and H_2O. They were then transferred into a M/2 KCl solution (instead of M/8, as in the experiments thus far mentioned). Table IV gives the results.

As in the preceding experiments we notice that the previous treatment with normal (M/2) sea water accelerates the diffusion of KCl more than a previous treatment with a M/2 Ringer solution. Again we notice that a moderate concentration of the Ringer solution, from M/16 to M/2, accelerates the diffusion more

than a higher concentration, like 5 M/2. The difference which is very striking during the first hours becomes less in time for two reasons; first, that the salt at the surface of the membrane diffuses into the solution, and second, that in as high a concentration as M/2 the KCl solution itself rapidly supplies the salt effect necessary for the diffusion of its own molecules or ions through the membrane.

The demonstration of the general salt effect in the case of the balanced solutions had the advantage of proving that this effect does not consist in an injurious action of the salt upon the membrane. Since M/2 sea water is the ideal balanced solution and the natural milieu in which these eggs develop, it cannot be said that eggs taken from normal sea water or from M/2 NaCl +

TABLE IV.

After	Percentage of eggs with beating hearts in M/2 KCl after having been kept for 17 hrs. in Ringer solution, sea water, or H_2O.												
	Ringer solution.											M/2 Sea water.	H_2O
	5M/2	4M/2	3M/2	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128		
hrs.													
2	90	80	90	65	20	30	65	85	100	95	85	5	100
4	60	30	30	25	15	15	55	70	95	95	85	0	100
6	35	10	5	15	10	10	45	65	95	85	75	0	85
8	15	0	0	15	5	10	30	45	70	70	55	0	75
12	5	0	0	5	0	5	5	30	50	30	35	0	50

$CaCl_2$ succumb more rapidly to KCl because the natural sea water hurts the membrane more than dilute sea water or H_2O. As a fact the contrary is more nearly correct.

Eggs were put for 24 hours into non-balanced solutions of various sodium salts of concentrations varying from M/1 to M/1,024 or less. They were then put into M/8 solutions of KCl. Only eggs with beating hearts were put into the KCl solution. Table V gives the percentage of beating hearts after 6 hours in the different solutions.

The difference in the efficiency of the anions in the production of the general salt effect is quite obvious. When we select for comparison the highest concentration of each salt which produced

TABLE V.

	Percentage of eggs with beating hearts in M/8 KCl after 6 hrs. when kept previously for 24 hrs. in												
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	M/2,048	H_2O
NaCl.....	0	0	0	16	100	100	100	100	100	100	100		100
NaBr.....	0	0	5	0	84	100	100	100	100	100	100	100	100
Na acetate...		0	0	30	80	100	100	100	100	100	100	100	100
Na_2SO_4.....		0	0	0	74	90	100	100	100	100	100		100
Na_3 citrate...								82	93	96	100	100	100
Na_2HPO_4....								42	67	100	100		100

no more salt effect, *i.e.*, that concentration which rendered 90 per cent of all the eggs immune against the M/8 KCl solution in the next 6 hours, we notice the following results:

NaCl.....M/ 16
NaBr.....M/ 32
Na acetate.....M/ 32
Na_2SO_4.....M/ 64
Na_3 citrate.....M/256
Na_2HPO_4.....M/512

Above these concentrations these salts produced the salt effect. The ratio of the limiting concentration for the salt effect of Na salts is therefore Cl : SO_4 : citrate or phosphate approximately as 1 : 4 : 16, the same as that found in the recovery experiments (second paper of this series).

It might be argued that the hearts stopped beating after the treatment with M/2 NaCl or NaBr or M/4 Na_2SO_4 not on account of the injurious effect of the M/8 KCl but on account of the toxic effects of the NaCl or Na_2SO_4 solutions. This argument would be wrong since only such eggs were transferred to the M/8 KCl solution whose hearts were beating after having been 24 hours in M/1, M/2 NaCl, or M/2, M/4, or M/8 Na_2SO_4, or any of the other solutions, and, moreover, we had found in the second paper that the relative efficiency of these salts to induce the recovery from potassium is exactly the same as that for poisoning in these experiments. Moreover we convinced ourselves that eggs which had been poisoned in M/8 KCl after a treatment in M/4 Na_2SO_4 recovered when put back into the latter solution although M/4 Na_2SO_4 rapidly kills the embryo outside the egg.

The effect of the cation is equally marked in Table VI, showing the efficiency of different chlorides. The eggs were put for 24 hours into the solutions of various concentrations of these salts; the concentrations were below that limit in which they kill or injure the egg in 24 hours. After 24 hours the eggs were put into an M/8 KCl solution. The percentage of eggs with heart beats is given in Table VI.

TABLE VI.

	Percentage of eggs with beating hearts in M/8 KCl after 6 hrs. which had previously been treated for 24 hrs. with										
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024
LiCl		70	85	47	89	90	100	100	100	100	100
NaCl	0	0	0	15	100	100	100	100	100	100	100
RbCl		33	88	96	100	100	100	100	100	100	100
CsCl			50	90	100	100	100	100	100	100	100
NH_4Cl			85	85	90	95	100	100	92	100	100
$MgCl_2$			85	100	100	100	100	100	100	100	100
$CaCl_2$				89	100	100	100	100	100	100	100
$SrCl_2$				96	100	100	100	100	100	100	100

This table reveals the unexpected fact that Na has the most striking salt effect of all the cations mentioned. Since NaCl is also the least toxic of all the salts and since it injures the membrane less than any of the other salts mentioned in the table, it is obvious that the acceleration of the diffusion of KCl through the membrane cannot be ascribed to an injurious effect of the salt on the membrane.

A previous treatment of the eggs with the solutions of non-electrolytes does not lead to an acceleration of the diffusion of the KCl through the membrane. Eggs were put for 24 hours into solutions of various non-electrolytes in different concentrations. Nothing comparable to the salt effect was produced; the eggs behaved as if they had been washed in H_2O.

It is obvious that the membrane of eggs which had been for 24 hours in a solution of a non-electrolyte, *e.g.*, glucose, cane sugar, glycerol, and methyl alcohol had become impermeable to M/8 KCl to the extent that in 6 hours not enough KCl could diffuse into the egg to cause cessation of the heart beat in more than 20 per cent of the eggs. The non-electrolytes act, therefore, essen-

TABLE VII.

	Percentage of eggs with beating hearts after treatment for 6 hrs. in M/8 KCl. Eggs previously kept for 24 hrs. in											
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	H_2O
Glucose.....		96	95	97	95	90	96	100	100	100		100
Cane sugar.....	81	96	100	100	100	100	100	100	100	100	100	100
Glycerol.....	81	90	90	90	95	96	100	100	100	100		100
Methyl alcohol...	84	100	100	100	100	100	100	100	100	100	100	100

tially like distilled water unless they are applied in excessive concentration. In that case we may expect an "injurious" action on the membrane, different both from the antagonistic action and the general salt effect upon the membrane. This phenomenon will be discussed in the fourth paper of this series.

II.

We shall now try to show that for the diffusion of acids through the membrane of the *Fundulus* egg, beside the osmotic pressure, a salt action upon the membrane is also required. The difference between the two cases is that acids can produce the salt effect in a concentration at least several hundred times smaller than the concentration required for the same purpose in the case of KCl. Thus M/1,000 acetic acid kills the egg rapidly by coagulating the embryo. In order to demonstrate the salt effect it was necessary to show that when the membranes of the egg were freed from salt by previous washing in H_2O, it took longer to kill the embryos than when eggs taken directly from sea water were exposed to the acid. The writer tried in vain last year to furnish this proof. He found this year the reason for the failure; he had washed the eggs for 24 hours in H_2O (as in the potassium experiments). This washing probably left a trace of salt on the external surface or the external layer of the egg membrane which was negligible for the diffusion of potassium salts but which was sufficient to prevent the demonstration of the salt effect in the case of acids. When eggs were washed for 4 days in H_2O it was possible to demonstrate the salt effect for acids too. The method of the experiment was as follows: Eggs were put for 4 days into H_2O and into different concentrations of NaCl + KCl

+ $CaCl_2$ (in the proportion of 100 molecules NaCl to 2.2 molecules KCl to 1.75 molecules of $CaCl_2$ as in sea water). They were then put into 50 cc. of M/1,000 acetic acid. In acid solutions the embryo is killed by coagulation and becomes white. The coagulation shows itself first in the tail of the embryo and then creeps upward. The heart is the last organ to be affected by the acid, probably because it is protected by the pericardium. Thus the tail of the embryo is already opaque when the heart ceases to beat. The cessation of heart beat in this case means death of the embryo. Table VIII gives the percentage of embryos whose hearts were still beating.

TABLE VIII.

After	Percentage of embryos surviving in M/1,000 acetic acid after having been kept for 4 days in the following solutions.											
	NaCl + KCl + $CaCl_2$										H_2O	M/2 Sea water.
	3M/2	M/1	M/2	M/4	M/8	M/32	M/128	M/512	M/1,024	M/2,048		
hrs.												
5	100	100	90	90	75	85	100	90	95	95	100	85
7	95	95	50	65	35	55	70	75	85	85	70	60
8	85	60	30	45	35	35	45	70	65	85	65	35
9	80	50	25	30	25	30	15	70	50	70	50	15
11	45	35	10	15	5	30	5	55	35	35	35	5
23	20	10	0	5	0	5	0	5	0	0	5	5

We must remember that the salt in combination with the surface from the previous treatment diffused during the first hours into the surrounding acid solution which was free from salts. We must therefore look for differences in the effects of the previous treatment of the eggs during the first hours of the experiment. After 5 hours all the embryos which had been in H_2O and 3 M/2 and M/1 Ringer were still intact in the M/1,000 acetic acid solution, while 25 per cent of those that had been in M/8 Ringer and 15 per cent of those that had been in sea water were already dead. After 7 hours two-thirds of those that had been in M/8 Ringer were already dead and not yet one-third of those which had been in distilled water had been killed. After 8 hours the accelerating salt effect upon the diffusion of acid through the membrane was still more striking; those that had been in

M/2 to M/32 Ringer or M/2 sea water died twice as fast as those that had been in H_2O. After 23 hours the effect of the previous treatment had disappeared since the salts had diffused from the surface of the membrane.

Hence we see that in the case of acids as in the case of KCl, a small dose of salt on the external surface of the membrane accelerates the diffusion, a larger dose retards the diffusion. In eggs washed in H_2O the acid itself supplies the salt effect upon the membrane, much less acid than neutral salt being required for this purpose.

On account of the theoretical importance of the subject, a second experiment of the same type may be reported. The eggs were put again for 4 days into the solution before being transferred to the M/1,000 acetic acid solution. Table IX gives the result.

TABLE IX.

After	Percentage of eggs with beating hearts in M/1,000 acetic acid after having been kept for 4 days in the following solutions.														
	NaCl + KCl + $CaCl_2$													H_2O	Sea water.
	5M/2	2M	3M/2	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512		
hrs.															
3	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
6	100	100	100	87	65	40	61	80	70	85	85	90	88	94	55
7	93	94	100	25	20	25	20	39	20	35	70	55	66	82	30
8	76	88	95	15	5	5	9	18	5	35	40	45	48	56	20
9	76	70	80	10	0	5	5	18	0	25	30	40	30	24	10
11	48	48	33	0	0	0	0	5	0	15	10	25	10	19	5
23½	15	6	14	0	0	0	0	0	0	5	0	5	5	0	0

A glance at the table will show that the eggs previously treated with a moderate concentration of salts, M/32 to M/1, or with sea water, are killed by the acid more quickly than the eggs previously treated with distilled water, while those treated with a higher concentration of the salt die more slowly (antagonistic salt action). These experiments show incidentally that the antagonistic salt action (or the general salt effect) occurs between the membrane (modified by the antagonistic salt) and the acid, and that it is immaterial whether the antagonistic salt is applied

simultaneously with the injurious salt or previously, except that the antagonistic salt action cannot last in the latter case on account of the diffusion of the salt from the membrane into the solution.

When the eggs were washed for 1 or 2 days only, the antagonistic salt action due to the previous treatment with the stronger solutions was more marked than the accelerating action of the more moderate salt concentrations as the following example shows (Table X).

TABLE X.

After	Percentage of eggs with beating hearts in M/1,000 acetic acid after having been kept for 2 days in the following solutions.															
	NaCl + KCl + $CaCl_2$														H_2O	Sea water.
	5M/2	2 M	3M/2	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024		
hrs.																
5	100	90	95	85	85	15	25	25	25	35	35	25	55	35	45	50
9	85	45	50	55	30	0	5	15	5	0	10	5	10	10	10	10
20	25	20	15	0	0	0	0	0	0	0	0	0	0	5	0	0

In this experiment the antagonistic effect of the salts (which were left on the surface of the membrane) on the diffusion of acid is very striking, especially in the eggs previously washed in 5 M/2, 2 M, and 3 M/2 NaCl + KCl + $CaCl_2$. A slight accelerating effect of the salt is only noticeable in the eggs previously washed in M/4 NaCl + KCl + $CaCl_2$. The washing was too short to remove enough salt from the surface of the egg in the H_2O so that the trace of salt left on the surface of the membranes of the eggs was still sufficient to supply the general salt effect. The diffusion of acids requires a much lower concentration of a second salt at the external surface of the membrane than the diffusion of KCl, possibly because acid forms a much more stable salt with certain proteins than neutral salts (Hardy); as a consequence, a trace of acid can by combining with the membrane (or its proteins) produce the same salt effect as a considerable concentration of a neutral salt.

We have already mentioned in a previous note[2] that the antagonistic action of salts to acid in the case of the *Fundulus*

[2] Loeb, *J. Biol. Chem.*, 1915, xxiii, 139.

embryos increases not only with the valency of the anion but with that of the cation as well, which is also the case when KCl is antagonized by other salts. We shall return to this fact in another connection, though we may add here that this should put an end to such generalizations as that the antagonistic salt action is due to the oppositely charged ions.

CONCLUSION.

This paper shows that under certain experimental conditions the existence of the general salt effect can be demonstrated also for the diffusion of acid through the membrane of the egg of *Fundulus*. The concentration of neutral salt required for the salt effect is considerably smaller in the case of the diffusion of acid than in the case of the diffusion of potassium salt. Very weak acid solutions themselves can supply the general salt effect, owing probably to the fact that acids form stable salts with certain proteins of the membrane (while neutral salts form only unstable salts).

When the concentration of neutral salt added to the acid is a little higher than that required for the salt effect the opposite phenomenon is produced; namely, the diffusion of acid is retarded or inhibited (antagonistic salt action).

It is shown that under the conditions of these experiments similar rules hold for the diffusion of potassium salts through the membranes of living eggs of *Fundulus*.

THE FATE OF ALKALI BLUE IN THE ORGANISM.

By SHIGENOBU KURIYAMA.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

(Received for publication, September 4, 1916.)

The excretion of lipoid-insoluble dyes after parenteral administration into frogs has been investigated by Höber and Kempner. They concluded that the more characteristically colloidal the state of the dye the more difficult is its passage through the kidneys. Alkali blue (*Alkali-blau*), which is a highly colloidal compound, was not eliminated through the kidneys.

The fate of various types of dyes in the organism is of interest in the study of the permeability and secretory power of blood vessels and glands. At the suggestion of Professor Lafayette B. Mendel, I have investigated the behavior of alkali blue in the organism. All necessary operations on animals were performed by him.

Methods.

Alkali blue—sodium triphenylrosaniline monosulfonate—is soluble in cold water, much more so in alcohol and in warm water, but insoluble in ether and in oil. It is precipitated by calcium or lead salts. The color is decreased by dilute ammonia, and it can be restored with acids. The dye (Kahlbaum's) used in the following experiments was dissolved in distilled water in the proportion of 1 or 2.5 per cent.

The method for the detection of the dye in tissues and in body liquids was investigated in preliminary experiments. The intensity of blue color decreases when the dye is passed through the animal body or kept a little while with body liquids. On addition of a small amount of acetic acid, the color reappears, but not always to the original intensity. Boiling or addition of hydrogen peroxide seemed to cause no improvement. Though the exact nature of the change is yet unknown, a similar altera-

tion of color has been noted with many other dyes. Underhill and Closson demonstrated that methylene blue, administered to an animal, reappears in the urine and feces in four forms—methylene blue itself, methylene azure, *i.e.*, an oxidation product of methylene blue, and leuko compounds corresponding to methylene blue and methylene azure. As these leuko compounds are reduction products of either methylene blue or methylene azure, methylene blue must undergo both oxidation and reduction in the animal body. Indigo carmine, rosaniline, and phenoltetrachlorphthalein, introduced into the organism, reappear in both free and leuko states. Bulk demonstrated that alkali blue becomes colorless by reducing agents, such as ammonium sulfide, the color being called forth again by oxidation.

For the detection of alkali blue in lymph, the material was slightly acidified with acetic acid, whereupon the blue color developed or deepened in a few minutes. In order to demonstrate the presence of the dye in blood, four volumes of 95 per cent alcohol were mixed with one volume of blood sample. The mixture was stirred vigorously, allowed to stand for 2 hours, and filtered. The filtrate was made faintly acid with acetic acid, whereupon the maximum color developed. For the detection of alkali blue in organs, tissues, or in dry feces, the material was ground in a mortar with 95 per cent alcohol in the proportion of 5 cc. alcohol for each gram thereof. The mixture was allowed to stand for 2 hours in a stoppered bottle. The filtrate was then treated as in the case of blood. To detect the dye in the urine, acetic acid was added without any other manipulation. The presence of the dye in the bile was easily demonstrated by acidifying. The result was especially clear when the bile was diluted with about 200 volumes of water before acid addition. In order to make an approximate estimation of the dye in the feces, the whole material was diluted to 1 or 2 liters. 50 cc. of it were mixed with neutral lead acetate, the latter being added until precipitation ceased. The sediment containing the whole amount of the dye was placed in a 100 or 250 cc. measuring flask, mixed with a few cc. of acetic acid, and made up to the mark with 95 per cent alcohol. The filtrate was then compared in a Duboscq colorimeter with the standard, prepared from the original dye solution by acidifying and diluting with alcohol.

PROTOCOLS.

Experiments with Dogs.

Experiment I.—Dog 1, male, 8.8 kg., was anesthetized with ether after injection of 90 mg. of morphine and 9 mg. of atropine. A cannula was introduced into the thoracic duct; and 8 cc. of 2.5 per cent solution of alkali blue were injected into the femoral vein.

TABLE I.

Lymph.

Administration of dye and diuretic.	Lymph flow in 10 min. intervals.		Blue color.	
	Sample No.	Amount.	Before acidification.	After acidification.
		cc.		
	Before injection.	4.2	−	−
8 cc. of alkali blue into femoral vein.				
	1	4.2	−	−
	2	2.9	−	+
	3	2.9	−	++
	4–7	2.8–4.4	−	++
8 cc. of alkali blue into femoral vein.				
	8–10	3.3–4.6	−	++
	11–12	4.0–4.6	+	+++
150 cc. of 0.9 per cent NaCl into femoral vein.				
	13–20	9.0–10.4	+	+++
	21–24	5.0– 7.4	+	+++

*Blood.**

Time after first dye injection.	Blue color.	
	Before acidification.	After acidification.
5 min.	±	+++
30 "	−	++
1 hr.	−	++
1 " 30 min.**	±	+++
3 hrs.	−	++
4 "	−	+

* Samples were taken from the femoral artery.

** 20 min. after the second injection of the dye.

Urine.—Samples, taken by aspiration of the bladder 1, 2, 3, and 4 hours after the first injection of the dye, did not contain the dye in a free form, but showed a very pale blue color when acidified with acetic acid. The urine flow was very slow.

Autopsy.—4 hours after the first injection of the dye, the dog was killed by bleeding. A strong blue color of the liver was the most noticeable feature observed. The other organs and tissues showed no such abnormal color. The alcohol extract of the liver appeared intensely blue. Acid addition made its color much brighter. The bile in the gall bladder contained the blue dye. The pyloric portion of the stomach mucosa was covered with intensely blue mucus, the other parts of the stomach showing the normal color. The blue color extended to the greenish blue mucus in the duodenum. The blue color of the latter was markedly increased by acidifying, but the color of the former showed no such intensification. The mucus in the upper part of the jejunum was brownish yellow and showed a slight blue color by acid addition. Further downwards such development of blue color by acid addition could not be demonstrated. Even where the stained mucus was contained the mucous membrane itself showed no abnormal color. The kidneys showed no evidence of blue color. In the alcohol extracts a blue color could not be observed, even after acidification.

Experiment II.—Dog 2, female, 7.7 kg. First a cannula was introduced in the thoracic duct, and before the conclusion of the experiment a bile fistula was established in the common bile duct. As narcotics urethane (10 gm.) and ether were used.

TABLE II.

Lymph.

Administration of dye and diuretic.	Lymph flow in 10 min. intervals.		Blue color.	
	Sample No.	Amount.	Before acidification.	After acidification.
		cc.		
	Before injection.	1.4	−	−
8 cc. of alkali blue into femoral vein.				
	1	1.4	−	±
	2	3.1	+	++
	3–5	2.9–5.4	+	+++
0.15 gm. of caffeine citrate and 100 cc. of 0.9 per cent NaCl into femoral vein.				
	6–11	2.0–4.8	+	+++
	12–18	0.7–1.6	+	+++

TABLE II.—*Concluded.*

*Blood.**

Time after the dye injection.	Blue color.	
	Before acidification.	After acidification.
5 min.	±	+++
50 "	−	++
1 hr. 50 min.	−	++
3 hrs.	−	+

* Samples were taken from the femoral artery.

Urine.—5, 85, and 180 minutes, respectively, after the dye injection, urine samples were obtained by catheterization. In none of them was the dye found. Though caffeine and saline solution were given, the urine secretion was very slow.

Bile.—3¼ hours after the dye injection, a cannula was inserted into the common bile duct. The bile flow in successive 10 minute periods was 0.9, 1.1, and 0.5 cc. The color of these samples was always dark blue. When kept a few hours, their color became yellowish green, but an addition of one drop of acetic acid called forth again the dark blue color.

Autopsy.—4 hours after the dye injection, the dog was killed by bleeding. The observations were essentially similar to those described for Dog 1, the exceptions being that the dye was not found in the stomach and duodenum, but it was abundant in the greater part of the jejunum and ileum.

Experiment III.—Dog 3, female, 9.5 kg. Cannulas were introduced into both the thoracic duct and the common bile duct, as in Dog 2. To induce a marked urine flow, a large amount of the saline solution was injected.

Urine.—Of the samples taken from time to time by catheterization, only one, removed 1½ hours after the dye injection, contained a trace of the dye, the others always yielding negative results. Though a large amount of the saline solution was injected, the urine flow was not augmented. Protein and casts were found in some samples.

Bile.—2 hours after the dye injection, a fistula was established in the common bile duct. The bile samples, collected during

TABLE III.

Lymph.

Administration of dye and diuretic.	Lymph flow in 10 min. intervals.		Blue color.	
	Sample No.	Amount.	Before acidification.	After acidification.
		cc.		
	Before injection.	4.8	−	−
8 cc. of alkali blue into femoral vein.				
	1* Part 1	2.0	−	−
	1* " 2	1.2	−	±
	1* " 3	0.8	−	+
	1* " 4	0.9	+	++
	2– 3	4.0–2.6	+	++
400 cc. of 0.9 per cent NaCl into femoral vein.				
	4– 5	3.3– 5.5	+	+++
	6–12	8.5–13.0	+	+++
	13–18	4.4– 8.0	+	+++

*Blood.***

Time after the dye injection.	Blue color.	
	Before acidification.	After acidification.
50 min.	−	++
1 hr. 30 min.	−	+
2 hrs.	−	+
2 " 30 min.	−	+
3 "	−	±

* Subdivisions were collected during each 2½ min.

** Samples were taken from the femoral artery.

successive 10 minute periods, measured 1.2, 1.3, 1.5, 1.0, 0.7, and 0.8 cc., respectively. The color was greenish blue and behaved as described for Dog 2.

Autopsy.—3 hours after the dye injection, the animal was killed by bleeding. The observations were essentially similar to those described for Dog 1. In the alimentary tract the dye was found in the pyloric portion of the stomach, the duodenum, and the jejunum.

Experiment IV.—Dog 4, male, 11.2 kg., was used to see whether alkali blue can enter the lymph otherwise than through the liver

lymphatics. As a narcotic ether was used. A cannula was placed in a cervical lymph duct. The lymph flow, facilitated by gentle massage, was very slow. 8 cc. of 2.5 per cent solution of alkali blue were injected into the femoral vein. The cervical lymph, taken within 10 minutes after the dye injection, showed no blue color, even by acid addition. The samples, taken later, changed to blue after acidification; without this manipulation the color was pale yellow. 30 minutes after the dye injection a lymph sample was taken from the thoracic duct. It had a pale green color, and after acid addition it developed an intense blue color, which was much deeper than that developed by acidifying the cervical lymph sample taken at the same time. This shows that the thoracic duct lymph, derived largely from the liver lymphatics, contained the dye in more concentrated form than the cervical lymph. 45 minutes after the dye injection the dog died suddenly. The bile, taken from the gall bladder, was yellow-brown and did not show any change of color by acid addition. Though the color change of the liver was not intense, an alcohol extract contained the dye in free form. The blood contained the dye only in the leuko state. The protein-free urine contained no dye.

Experiment V.—Dog 5, male, 9.5 kg., was used for comparison, phenoltetrachlorphthalein and indigo carmine being the dyes injected. After a dose of morphine (95 mg.) and atropine (9.5 mg.), the dog was anesthetized with ether.

Urine.—The sample, taken $1\frac{1}{2}$ hours after the phenoltetrachlorphthalein injection, became faintly red by NaOH addition. The samples, taken 30 and 70 minutes after the indigo carmine injection, were so dark blue with the dye that they could not be tested for the red dye.

Autopsy.—The mucous membrane of the mouth was markedly blue. All viscera were stained a blue color. The liver was also blue, but not so intense as seen in the experiments with alkali blue. The bile in the gall bladder was markedly blue. The stomach mucosa showed nothing abnormal. The duodenum contained blue mucus. Its color became a little stronger by acid addition. Moreover, with NaOH addition a strong red color appeared. The first part of the jejunum contained brownish yellow mucus. No color change was seen by acidification, but a slight red color developed after alkali addition. Further down no color change was produced either by acid or alkali addition.

TABLE IV.

Lymph.

Administration of dye.	Lymph flow in 10 min. intervals.		Red color.		Blue color.	
	Sample No.	Amount.	Before alkali addition	After alkali addition.	Before acid addition.	After acid addition.
		cc.				
	Before injection.	1.8	−	−		
8 cc. of 2.5 per cent phenoltetrachlorphthalein into femoral vein.						
	1	3.5	−	±		
	2	1.4	−	+		
	3–14	1.1–5.4	−	++		
20 cc. of 1 per cent indigo carmine into femoral vein.						
	15	8.0			+*	++
	16	8.0			++	+++
	17–21	4.8–9.6			++	+++

Blood.†

Time after phthalein injection.	Red color.		Blue color.	
	Before alkali addition.	After alkali addition.	Before acid addition.	After acid addition.
10 min.	−	++		
50 "	−	+		
2 hrs. 10 min.	−	±		
2 " 40 " **	−	±	−	+
3 "	−	−	−	−

* The first appearance of the dye in a free state was 6 min. after the dye injection.

† Samples were taken from the femoral artery.

** 20 min. after indigo carmine injection.

Experiment VI.—Dog 6, male, 5.0 kg., was used to see whether alkali blue is eliminated into the alimentary tract otherwise than through the bile duct.

The day before the operation no food was given. As narcotics urethane (3.8 gm.) and ether were used. A temporary bile fistula was made in the common bile duct. The bile flow was very slow, only 0.1 cc. being collected in 30 minutes. 5 cc. of 2.5 per cent solution of alkali blue were injected into the femoral vein. Bile samples were collected during successive 30 minute periods. No dye was found in Sample 1 but subsequent samples were all intensely blue. The color was greatly increased by acid addition. The properties of the bile were the same as those described for Dog 2. The bile flow became rapid at first and then again slow, as shown by the volumes of the successive bile samples measuring 0.1, 0.2, 0.2, 0.7, 1.9, 0.8, 0.2, 0.2, 0.2, 0.2, and 0.2 cc., respectively.

Autopsy.—6 hours after the dye injection the animal was killed by bleeding. The contents and the mucous membrane of the alimentary tract showed no blue color, even after acid addition. The submucous tissue of the alimentary tract, especially of the stomach, had a pale blue color, though its alcohol extract did not show any blue tone. Alcohol extracts of various organs and tissues, such as kidneys, spleen, mucous membrane of the alimentary tract, blood, and mediastinal and mesenteric lymph glands, failed to show blue color. Only the liver and the bile in the gall bladder contained the dye in a large amount. No dye was found in the urine; but protein and casts were present.

Experiment VII.—To induce marked flow of urine, Dog 7, female, 14 kg., was made diabetic by phlorhizin. 8 cc. of 2.5 per cent solution of alkali blue were then injected into the jugular vein. The amount of the urine in 24 hours after the dye injection was 1.6 liters. In spite of this diuresis, no dye was eliminated through the kidneys; but an alcohol extract of the feces showed the presence of the dye.

Experiment VIII.—Dog 8, male, 8.2 kg., was used to see to what extent alkali blue, injected intravenously, will be recovered in the feces. 8 cc. of 2.5 per cent alkali blue solution were injected into the jugular vein. In order to cause discharge of feces, magnesium sulfate was administered by a stomach sound 3 and 20 hours respectively after the dye injection. The feces in 24 hours after the injection contained about 8 per cent of the dye; during the next 24 hours, about 5 per cent. On the 3rd day only a trace of the dye was found in the feces.

Experiment IX.—Dog 9, female, 9.5 kg., was used to see to what extent alkali blue, administered by mouth, will be recovered in the feces. 8 cc. of 2.5 per cent alkali blue solution were given by a stomach sound. To induce purgation, magnesium sulfate (25 and 10 gm.) was administered into the stomach, immediately and again 20 hours after the dye administration. The feces in 24 hours after the dye administration contained 58 per cent of the dye, given by mouth.

Experiments with White Rats.

The body weight of the rats varied from 170 to 310 gm. In Rats 1 and 2, 1 cc. of 1 per cent alkali blue solution, in Rats 3 and 4, 1.5 cc. of 2.5 per cent alkali blue solution were injected intraperitoneally. No change of the urine flow and no elimination of the dye were observed. The dye was always present in the feces. 30 hours after the dye injection, the animals were killed. The peritoneum, especially the omentum, and pleuræ were intensely blue in color, but no dye was found in solution in the cavities. The liver appeared greenish blue. Mesenteric and mediastinal lymph glands were also stained with the dye. In the blood and the remaining viscera the dye could not be demonstrated. The test for it was positive in the intestinal contents. In Rat 5, for contrast, 1 cc. of 1 per cent indigo carmine solution was injected intraperitoneally. The dye was eliminated very rapidly through the kidneys and no trace of it was found in the feces. 48 hours after the dye injection the animal was killed. The dye could nowhere be demonstrated. In Rats 6 and 7, phlorhizin (0.05 and 0.1 gm.) was injected subcutaneously, to induce marked flow of urine. 1 cc. of 25 per cent alkali blue solution was injected intraperitoneally. Though the urine flow was greater than with the control animal, the dye was not eliminated through the kidneys, but was present in the feces. In Rats 8 and 9, 1 cc. of 2.5 per cent alkali blue solution was injected subcutaneously. The dye test was negative in the urine, but positive in the feces. The blue color at the injection area was very strong even after 1 month. In Rats 10, 11, and 12, powdered alkali blue was administered by mouth three times a day—about 0.05 gm. at a time. The feces were blue, but

the urine contained no dye. After 2 or 3 days the animals were killed. The dye was found in the alimentary tract only, the alcohol extracts of the liver, kidneys, spleen, and blood always yielding negative results.

Experiments with Rabbits.

Experiment I.—Rabbit 1, 1.9 kg. 2 cc. of 2.5 per cent alkali blue solution were injected into an ear vein.

TABLE V.

*Blood.**

Time after the dye injection.	Blue color.	
	Before acidification.	After acidification.
hrs.		
1	−	++
2	−	+
3	−	+
4	−	−

* Samples were taken from an ear vein of the other side.

The *urine* contained neither dye nor protein. The dye test was positive in the *feces*. (Diarrhea was induced by a purgative.)

Experiment II.—Rabbit 2, 1.6 kg. 1.5 cc. of 2.5 per cent alkali blue solution were injected into the jugular vein under cocaine local anesthesia. The result was the same as in Rabbit 1, except that the dye could not be demonstrated in the blood 3 hours after injection.

DISCUSSION.

Passage of Alkali Blue from Blood into Lymph.—Alkali blue, injected intravenously, appears very rapidly in the lymph of the thoracic duct—in a leuko form within 10 minutes, in a free form a few minutes later (Dogs 2 and 3). The delayed appearance of the dye in Dog 1 may have been due to the use of morphine and atropine which affect the permeability of the cellular membranes. Although the dye appeared also in the cervical lymph, its concentration was much less than in the thoracic lymph (Dog 4). Phenoltetrachlorphthalein and indigo

carmine, injected intravenously, appeared in the thoracic lymph within 10 minutes. The passage of the latter dye seemed to be much easier than that of the former (Dog 5).

Disappearance from the Circulation.—In the dog experiments, blood samples taken 3 or 4 hours after the intravenous injection of alkali blue (21 to 26 mg. per kilo), contained the dye (Dogs 1, 2, and 3), but a sample taken after 6 hours showed a negative result (Dog 6). In the rabbit experiments the dye disappeared from the circulation in 3 to 4 hours after its injection. Phenoltetrachlorphthalein, on the other hand, could not be demonstrated 3 hours after its injection. Indigo carmine was not detected in the circulation 70 minutes after its injection (Dog 5). This difference in the rapidity of passage of the three unlike dyes from the circulation is characteristic. Keith and his collaborators showed that vital red remains in the circulation for a long time. The time needed for elimination from the blood vessels thus varied markedly, according to the properties of the dyes.

Effect upon the Lymph Flow.—The rate of lymph flow was not markedly changed after the alkali blue or phenoltetrachlorphthalein administration (Dogs 1, 2, 3, and 5). On the contrary, indigo carmine or physiological saline solution caused a rapid lymph flow (Dogs 1, 3, and 5).

Elimination in the Bile.—Evidence was obtained that the liver is the chief if not the only organ for the elimination of alkali blue. Alkali blue, injected parenterally (intravenously, intraperitoneally, or subcutaneously), was always found abundantly in the liver and in the bile. About 30 minutes after intravenous injection of alkali blue, the dye began to appear in the bile (Dog 6).

Effect upon the Bile Flow.—As seen in Dog 6, the bile flow increased markedly after the alkali blue injection, the maximum being $2\frac{1}{2}$ hours after the dye injection. Though the effect of the narcotics or other factors may be of significance here, it is not improbable that the dye, which is chiefly eliminated into the bile, may of itself influence the rate of biliary flow. Abel and Rowntree, who investigated the effect of phenoltetrachlorphthalein upon the bile flow in an anesthetized dog, noticed no marked influence, the variations in rate being inconstant.

Elimination of Dye in the Urine.—In none of the experiments were more than traces of alkali blue eliminated in the urine. In

some experiments with dogs a decrease of the urine flow was noticed. Protein and casts were also found. Ordinary diuretics were not effective. In addition to a possible toxicity the colloidal condition of the dye may have had some relation to the decrease of the urine flow, as Spiro and Buglia reported for gelatin or gum arabic. Even in the phlorhizinized animals (Dog 7, and Rats 6 and 7), which showed a profuse flow of urine, the dye was also absent from the urine. Indigo carmine, on the other hand, was always found in the urine in a very high concentration (Dog 5 and Rat 5). As alkali blue is a derivative of aniline the *p*-aminophenol test was performed with the urine. The results were always negative. Rosaniline (rosaniline trisulfate of soda) has been used as a functional test of kidneys by Lépine, Dreyfus, and others. It is very easily eliminated through the kidneys. The difference in behavior between rosaniline and alkali blue is somewhat similar to the difference between phenolsulfonephthalein and phenoltetrachlorphthalein.

Absorption and Elimination from the Alimentary Tract.—Experiments with Rats 10, 11, and 12 showed that alkali blue is not absorbed from the alimentary tract. Abel and Rowntree showed that phenoltetrachlorphthalein and phenolsulfonephthalein, eliminated in the bile, could be reabsorbed from the alimentary tract, the former from the large intestine, the latter from the whole intestine. No evidence similar to this was gained with the colloidal alkali blue. When alkali blue was administered by mouth and a purgative was given afterwards, 58 per cent of the dye was detected in the feces (Dog 9). But, when the dye was injected intravenously, its recovery in the feces was not so successful as has been reported for phenoltetrachlorphthalein (Rowntree's test for liver function). Part of the alkali blue seems to be changed into some undetected forms in the tissues and in the alimentary tract. This was also demonstrated *in vitro*. A mixture of the dye and feces emulsion loses its dye content gradually. Alkali blue was not eliminated from the mucous membrane of the alimentary tract, as shown in Dog 6, in which the bile was prevented from pouring into the intestine.

Absorption from the Subcutaneous Tissues.—Part of the alkali blue injected subcutaneously remained for a long time under the skin. In Rats 8 and 9 the area of the injection was intensely

blue even after a month. In this respect, alkali blue is much different from phenoltetrachlorphthalein, which Abel and Rowntree could not find at the site of injection 16 to 24 hours after a subcutaneous administration.

SUMMARY.

Alkali blue, injected intravenously, rapidly appears in the lymph in both leuko and free states. In the cervical lymph it is less concentrated than in the thoracic duct lymph. The lymph flow is not affected markedly by the dye injection. Alkali blue, injected intravenously in doses of 21 to 26 mg. per kilo, remains in the circulation for a few hours only.

When administered parenterally, alkali blue is chiefly eliminated in the bile. The dye seems to act somewhat as a cholagogue. It is not eliminated by the kidneys except in traces. No evidence of its elimination or reabsorption through the alimentary tract was gained. When administered by mouth, the dye is not absorbed and more than a half of the given amount may be recovered in the feces with methods at present available. After intravenous injection the recovery of the dye in the feces is not so successful as has been reported for the somewhat comparably functioning phenoltetrachlorphthalein. The elimination of alkali blue through the liver seems to be rather slow and part of the dye is changed into some as yet undetected form in the alimentary tract. Absorption of the dye from connective tissue spaces is very tardy. These facts correspond with what might now be expected with a dye of the colloidal properties noted.

I desire to express my thanks to Professor Lafayette B. Mendel for his suggestions and criticism, also to Professor Frank P. Underhill for his help.

BIBLIOGRAPHY.

Abel, J. J., and Rowntree, L. G., *J. Pharm. and Exp. Ther.*, 1909–10, i, 231.

Buglia, G., *Biochem. Z.*, 1910, xxiii, 215.

Bulk, C., *Ber. chem. Ges.*, 1872, v, 417.

Höber, R., and Kempner, F., *Biochem. Z.*, 1908, xi, 105.

Keith, N. M., Rowntree, L. G., and Geraghty, J. T., *Arch. Int. Med.*, 1915, xvi, 547.

Okada, S., *J. Physiol.*, 1914–15, xlix, 457.

Rowntree, L. G., and Geraghty, J. T., *J. Pharm. and Exp. Ther.*, 1909–10, i, 579.

Rowntree, L. G., Hurwitz, S. H., and Bloomfield, A. L., *Bull. Johns Hopkins Hosp.*, 1913, xxiv, 327.

Rütimeyer, L., *Arch. exp. Path. u. Pharm.*, 1881, xiv, 393.

Spiro, K., *Arch. exp. Path. u. Pharm.*, 1898, xli, 148.

Underhill, F. P., and Closson, O. E., *Am. J. Physiol.*, 1905, xiii, 358.

Whipple, G. H., Mason, V. R., and Peightal, T. C., *Bull. Johns Hopkins Hosp.*, 1913, xxiv, 207.

EXPERIMENTAL STUDIES ON GROWTH.

VIII. THE INFLUENCE OF A DIET DEFICIENT IN FATS, AND OF THE SAME DIET WITH CHOLESTEROL ADDED, UPON THE GROWTH OF THE WHITE MOUSE.

By T. BRAILSFORD ROBERTSON.

(*From the Department of Biochemistry and Pharmacology, the Rudolph Spreckels Physiological Laboratory, University of California, Berkeley.*)

(Received for publication, August 29, 1916.)

The experiments which are about to be described were originally undertaken with a view to determining the effect upon growth of a diet, otherwise as normal as possible, which is practically cholesterol-free. Such a diet, however, if composed of natural foodstuffs, was found to be necessarily almost lipoid-free, so that the effects observed may be, and in view of the failure of addition of cholesterol to neutralize these effects, probably are, attributable to the absence of other lipoids, or substances commonly associated with lipoids. The experiment therefore failed to accomplish its immediate purpose and the results obtained, as will be seen, merely afford a confirmation of the results previously reported by a number of investigators.[1] Since this confirmation was reached from a widely divergent angle, however, it appears to be worth reporting as affording a wider foundation for the view of the necessity of lipoids in the diet which has found general acceptance in consequence of the observations cited above.

[1] Stepp, W., *Biochem. Z.*, 1909, xxii, 452; *Z. Biol.*, 1912, lvii, 135; 1913, lxii, 405. Mendel, L. B., and Osborne, T. B., *J. Biol. Chem.*, 1912, xii, 81; 1912–1913, xiii, 233; 1913–1914, xvi, 423. McCollum, E. V., Halpin, J. G., and Drescher, A. H., *ibid.*, 1912–13, xiii, 219. McCollum, E. V., and Davis, M., *ibid.*, 1913, xv, 167. Funk, C., *J. Physiol.*, 1911–12, xliii, 395; 1912, xliv, 50. Cooper, E. A., *Biochem. J.*, 1914, viii, 250. Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425. MacArthur, C. G., and Luckett, C. L., *J. Biol. Chem.*, 1915, xx, 161.

The diet administered consisted of boiled, peeled, and mashed potato, mixed for roughage with a small proportion of bran which had been defatted by extraction for 24 hours with hot alcohol followed by extraction for 24 hours with ether. This item of the diet was supplied *ad libitum*, water was also supplied *ad libitum*, and in addition to these items every group of six mice received daily (except Sundays) 10 cc. of white of egg to which were added five drops of Merck's water-soluble chlorophyll containing 0.2 per cent ferric chloride, equivalent to about 0.1 mg. of ferric chloride per mouse per day.[2]

In the case of the mice receiving cholesterol, 0.5 gm. of Merck's crystalline cholesterol was rubbed up with the 10 cc. of white of egg supplied to each six mice.

The diet of the animals which did not receive the added cholesterol was cholesterol-free, since Ellis and Gardner have found that neither potato nor white of egg contain any trace of cholesterol.[3] On the other hand, however, the diet was also very nearly fat-free, since white of egg contains only traces of fats, soaps, or phospholipins[4] and potatoes boiled in their skins contain only 0.03 per cent of fat.[5]

In other respects the treatment and manipulation of the mice were identical with the procedure previously reported.[6] Twenty-four mice of each sex and about 4 or 5 weeks of age were taken at random[7] and fed thereafter with the lipin-deficient diet. A similar number of males were similarly selected and fed thereafter with the lipin-poor diet plus cholesterol. The results are summarized in Tables I, II, and III and depicted graphically in Figs. 1, 2, and 3. In computing the average weights and

[2] A small number of the mice did not receive the chlorophyll and iron for several weeks. No difference due to this omission was observed in the symptoms displayed.

[3] Ellis, G. W., and Gardner, J. A., *Proc. Roy. Soc., Series B*, 1913, lxxxvi, 14.

[4] Hammarsten, O., Text-book of Physiological Chemistry, 6th edition, New York, 1911, 601.

[5] Williams, K. J., *J. Chem. Soc.*, 1892, lxi, 239.

[6] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxiv, 347.

[7] In replacing animals which died within 1 or 2 weeks of initiating the diet large animals were purposely selected. Hence the initial average weight of the survivors was, in each experimental group, supernormal.

variabilities the weights for 5 weeks preceding the death of any individual were rejected excepting after the 28th week.

TABLE I.

Male Mice Fed on Fat-Deficient Diet. Mean Duration of Life 27.2 Weeks. Variability of Duration of Life 37 Per Cent.

Age.	Weight.		No. weighed (fat-deficient diet).
	Normal.	Fat-deficient diet.	
wks.	*gm.*	*gm.*	
4	12.38	15.29	9
5	12.45	14.31	16
6	15.58	14.41	17
7	18.08	15.09	17
8	19.36	15.00	15
9	20.63	15.17	15
10	21.19	16.08	13
11	21.81	16.58	13
12	22.65	16.73	13
13	23.31	17.27	13
14	23.96	18.29	12
15	24.28	18.96	12
16	24.75	19.13	12
17	25.21	20.64	11
18	25.61	21.50	11
19	25.81	21.91	11
20	26.10	21.91	11
21	26.28	21.68	11
22	26.06	22.15	10
23	26.34	21.70	10
24	26.82	21.40	10
25	27.05	20.85	10
26	26.94	20.65	10
27	26.55	20.15	10
28	27.19	19.80	10
29	27.08	18.55	10
30	27.23	22.33*	6

* The apparent rise in weight at this point is due to the lightest animals having succumbed.

From the first the animals failed to do well on this diet. A large mortality occurred during the first few weeks and a majority of the animals were afflicted with diarrhea during this period. This rendered it a very difficult matter to distinguish with cer-

TABLE II.

Female Mice Fed on Fat-Deficient Diet. Mean Duration of Life 27.8 Weeks. Variability of Duration of Life 33 Per Cent.*

Age.	Weight.		No. weighed (fat-deficient diet).
	Normal.	Fat-deficient diet.	
wks.	*gm.*	*gm.*	
4	10.39	13.17	6
5	11.81	12.03	17
6	14.12	11.80	22
7	16.77	12.38	20
8	17.99	12.85	20
9	18.78	12.70	20
10	19.38	13.22	18
11	20.04	13.79	17
12	20.31	13.59	17
13	21.04	13.94	17
14	21.21	14.38	17
15	21.78	15.41	17
16	22.14	15.68	17
17	22.29	16.06	17
18	22.22	16.74	17
19	22.60	17.03	17
20	22.60	17.30	15
21	23.06	17.07	15
22	23.32	17.21	14
23	23.51	17.18	14
24	23.68	17.15	13
25	23.79	16.73	13
26	24.04	16.35	13
27	24.00	16.35	13
28	23.58	15.85	13
29	23.84	15.14	11
30	23.92	15.60	10

* Except the female which was fed after the 44th week upon tethelin and which survived to the age of 64 weeks.

tainty the animals affected with β-paratyphoid while, on the other hand, had every animal displaying diarrhea been sacrificed, nearly all of the experimental material would have been lost. As an approximate method of evading this difficulty, blood-stained feces or exceptionally severe diarrhea were held indicative of β-paratyphoid, and animals displaying these symptoms

TABLE III.

Male Mice Fed on Fat-Deficient Diet Plus Cholesterol. Mean Duration of Life 22.2 Weeks. Variability of Duration of Life 41 Per Cent.

Age.	Weight.		No. weighed (fat-deficient diet + cholesterol).
	Normal.	Fat-deficient diet + cholesterol.	
wks.	*gm.*	*gm.*	
4	12.38	11.42	6
5	12.45	12.96	14
6	15.58	13.45	21
7	18.08	14.10	20
8	19.36	15.00	17
9	20.63	15.53	15
10	21.19	15.46	13
11	21.81	15.65	13
12	22.65	16.54	12
13	23.31	16.67	12
14	23.96	17.04	12
15	24.28	18.42	12
16	24.75	20.05	11
17	25.21	19.95	11
18	25.61	19.27	11
19	25.81	19.70	10
20	26.10	20.64	7
21	26.28	20.50	7
22	26.06	19.79	7
23	26.34	21.00	6
24	26.82	20.58	6
25	27.05	20.08	6
26	26.94	20.00	6
27	26.55	19.16	6
28	27.19	19.00	6
29	27.08	18.50	6
30	27.23	18.08	6

were killed and replaced by others, the cage and food and water containers being washed down with alcohol.

During this period a decided loss of weight was suffered by the animals receiving the lipin-poor diet alone. The animals receiving this diet with the addition of cholesterol, however, showed no loss of weight, save in a few individual cases, but, on the contrary, a decided gain from the very first (compare Tables I and III). It is difficult to interpret this very striking difference

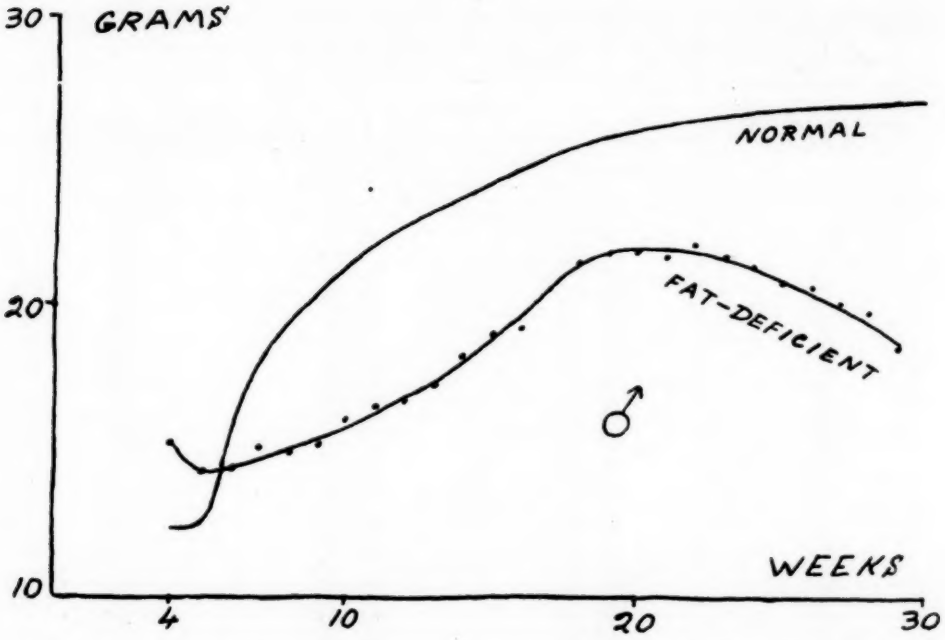


FIG. 1. Comparison of the growth curves of normal males and of males fed upon a diet deficient in fats.

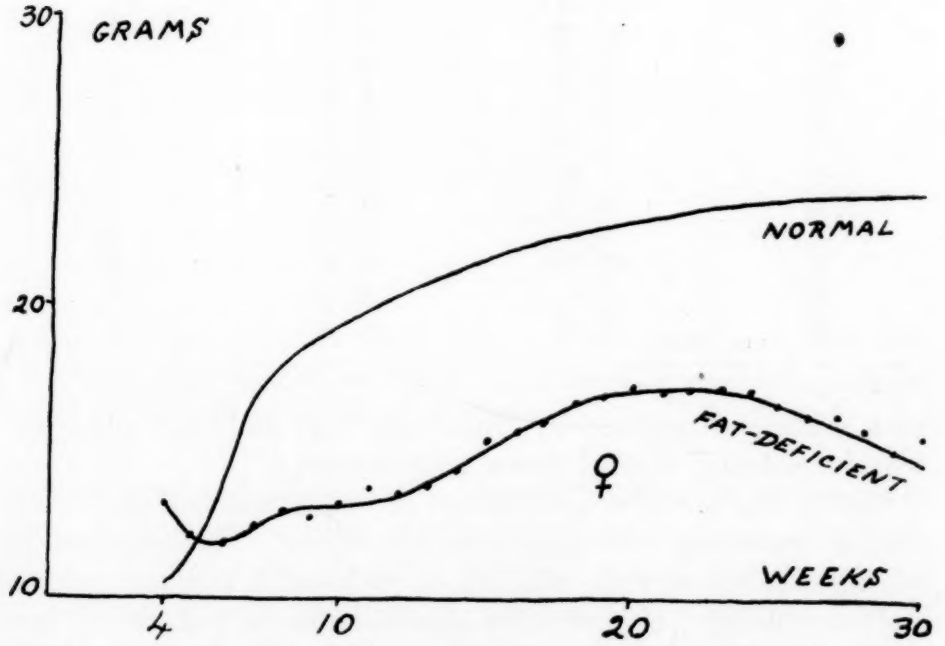


FIG. 2. Comparison of the growth curves of normal females and of females fed upon a diet deficient in fats.

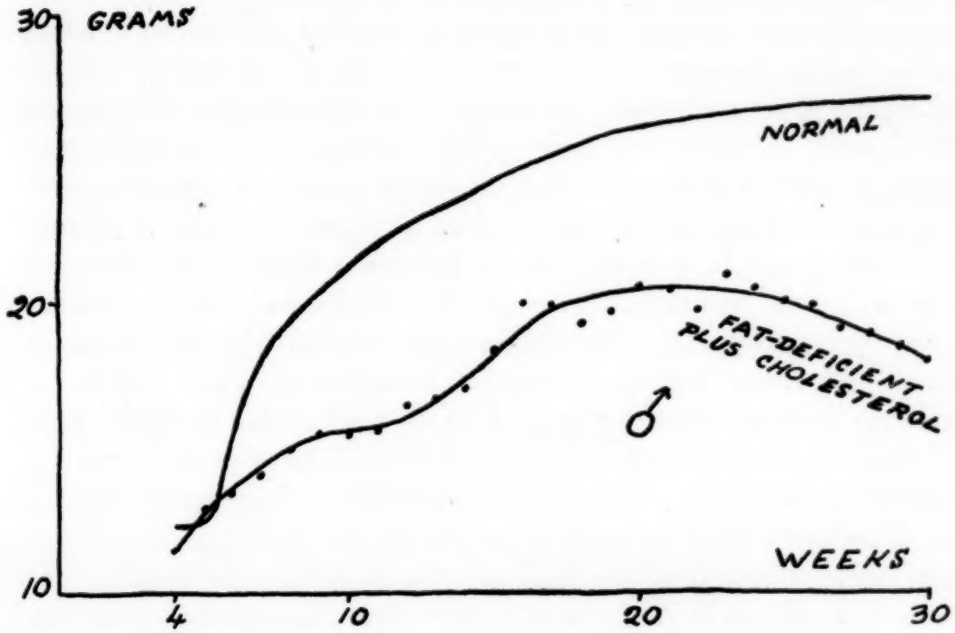


FIG. 3. Comparison of the growth curves of normal males and of males fed upon a diet deficient in fats, to which cholesterol has been added.

between the behavior of the two groups of animals, more especially as the animals receiving cholesterol displayed no such superiority in the later weeks of the experiment and actually survived for a shorter period than the animals which did not receive cholesterol.

In addition to diarrhea and loss of weight, the animals fed upon the lipin-free diet also displayed, during the first few weeks after the initiation of the diet, the following symptoms: The tails were cold, clammy, and pale, and there was a decided tendency to develop gangrene in the tip of the tail, with resultant loss of several joints. The ears were perfectly white, the penis was usually extended and became inflamed and frequently gangrened. The rectum appeared inflamed. The movements of the animals were languid and they were notably lacking in strength. The animals receiving cholesterol also displayed these symptoms, but in a milder degree.

At 8 or 9 weeks of age, that is, about a month or 5 weeks after the initiation of the diet, the condition of the animals noticeably

improved although it was at no period remarkably good. They then gained in weight, the lesions in the tail and penis healed, and the appearance of the coat improved. A fairly steady although slow accretion of weight continued until about the 22nd week (a highly variable period in the different individuals) when a sudden and rapid loss of weight occurred, accompanied by marked hyperirritability of the skin resulting in incessant scratching and in a few cases in punctate effusion of blood at the surface of the skin. The movements became weak, tremulous, and uncertain. The animals ate incessantly, and in some cases the urine had a markedly ammoniacal odor. After a varying period, usually about 4 or 5 weeks, the animals died, sometimes after several convulsions of brief duration followed by apparent recovery. The animals receiving cholesterol showed no superiority over the animals receiving the lipin-free diet alone, and their duration of life was, as will be seen, even briefer. It is a fact which is perhaps not devoid of significance that the final rapid loss of weight and incidence of death occurred in each group of animals at an average age approximately coinciding with a period of relative instability in the growth of normal animals.[8]

The duration of life in each group was highly variable and some individuals even survived for over 40 weeks. One of these individuals (a female) at 44 weeks had already lost 2 gm. in the preceding month and by analogy with the other animals had only a few days to live. 4 mg. per day of tethelin were now administered to this animal, with the white of egg. The lost weight was slowly regained during the succeeding month and this animal attained the age of 64 weeks. The usual sharp drop in weight and hyperirritability made their appearance at the 60th week, however, and the symptoms preceding death did not differ in any noticeable fashion from those displayed by the other animals. Whether the great prolongation of life in this individual, as compared with the other members of its group, was attributable to the administration of tethelin or not, cannot of course be decided without more extended investigation.

From these results it is evident that although growth is possible

[8] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 372.

on the diet described, it is impossible to maintain beyond a certain period the tissue which has been gained. In fact, paradoxical as it may appear, we have here a diet upon which, in contrast to certain diets lacking particular amino-acids,[9] growth is possible but maintenance is not.

The influence of these diets upon the variability of the animals to which they were fed is summarized in Table IV. In each

TABLE IV.

Variability.

Age.	Males.			Females.	
	Normal.	Fat-deficient diet.	Fat-deficient diet + cholesterol.	Normal.	Fat-deficient diet.
wks.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
4	24.4	19.6	14.2	23.4	12.0
5	24.6	18.2	19.8	19.4	16.0
6	22.0	20.3	16.6	18.9	15.4
7	16.9	21.8	20.1	15.0	16.4
8	15.9	22.9	19.6	13.9	17.9
9	16.5	24.9	18.2	13.4	20.9
10	16.7	21.3	20.7	14.3	20.2
11	13.3	23.6	20.8	12.6	23.4
12	13.7	21.3	21.9	12.6	18.2
13	14.2	23.3	20.3	13.0	20.2
14	14.1	23.9	20.9	13.2	19.9
15	11.9	22.8	21.6	13.7	22.6
16	12.7	22.5	15.2	12.6	22.1
17	12.4	19.9	15.0	11.9	22.7
18	12.1	20.8	15.9	11.9	22.5
19	12.1	19.7	19.2	12.0	23.7
20	10.8	17.8	19.4	11.2	22.7
21	11.2	18.9	20.3	12.3	23.4
22	9.0	18.3	21.3	12.5	18.7
23	9.8	20.3	13.6	12.0	19.3
24	10.1	20.4	13.7	11.3	22.9
25	11.0	20.8	17.1	11.4	21.9
26	10.8	23.0	18.1	11.6	22.1
27	11.3	24.6	19.8	11.8	23.1
28	10.9	27.0	20.4	10.3	24.0
29	10.9	29.2	20.1	12.2	26.5
30	9.5	14.4	25.7	12.4	27.4

[9] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325.

instance the initial variability of the animals was subnormal. Yet in a very brief period of feeding upon the diets the variability became supernormal and instead of decreasing with slackening growth, as in the normals, maintained a high level throughout. This would probably be found to be a general effect of adverse conditions of life, since unfavorable dietetic or environmental conditions might be expected to enhance preexisting deviations from the mean, initially supernormal animals resisting the pressure of the unfavorable environment more successfully than the average and thus departing more widely than before from the now subnormal average, while initially subnormal animals may be expected to become more subnormal than ever.

SUMMARY.

1. A diet composed of boiled and mashed potatoes, defatted bran, and white of egg, with the addition of small amounts of chlorophyll and ferric chloride, when fed to mice of 4 or 5 weeks of age, leads to initial loss of weight followed by resumption of a retarded growth. Ultimately, however, a sharp decline in weight occurs, accompanied by marked hyperirritability of the skin and terminated by death.

2. The addition of cholesterol to the above diet prevents the initial loss of weight, but does not otherwise improve the welfare of the animals, the average duration of life of the animals receiving cholesterol being actually less than that of the animals which do not receive cholesterol.

3. It follows that although growth is possible on the diet described, maintenance of the tissue gained is not. Decline of weight therefore occurs at an age when the diminishing velocity of growth has become insufficient to compensate for the deficient ability of the tissues to replace their current loss.

4. The variability of animals fed upon the diets described, as is probably to be expected under any unfavorable environmental or dietetic conditions, instead of falling with increasing age and slackening of growth, maintains a high level throughout the life of the animals.

PHYSIOLOGICAL AND PHARMACOLOGICAL STUDIES ON COAL TAR COLORS.

I. EXPERIMENTS WITH FAT-SOLUBLE DYES.

By WILLIAM SALANT and ROBERT BENGIS.

(*From the Pharmacological Laboratory of the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

(Received for publication, September 7, 1916.)

Although synthetic dyes have played an important part in the study of biology and medicine, the behavior of many of these compounds in the body is still imperfectly understood and the action of some of them is entirely unknown. Owing to their extensive employment in numerous industries, and especially in the preparation of foods, this lack of satisfactory information frequently proved to be a matter of serious import as questions regarding their effect on health were often raised, but no definite answer could be given in the present state of our knowledge.

The recognition of the need of a more intimate acquaintance with the reactions produced in the body by these compounds formed the starting point of various experimental inquiries in this laboratory, the present report being the first of a series of communications embodying the results of some of these investigations.

Previous observations on the changes which might occur in fat-soluble dyes within the body or on the effects produced by them have been confined to only a few members of this group and were limited in scope. The experiments reported during the past two decades by Daddi (1), Biedermann (2), Sitkowski (3), Hofbauer (4), Riddle (5), S. H. and S. P. Gage (6, 7), Mann (8), and others dealt chiefly with selective action and occasionally with the path of elimination, benzeneazobenzeneazo-β-naphthol (Sudan III) being largely employed in these studies. But the more recent investigations on this subject,

notably those of Mendel and Daniels (9), included attempts to gain information concerning the mode of absorption, distribution, elimination, and also toxicity which were carried out with a number of fat-soluble dyes, including Sudan III.

The present report embraces the results of experiments with fat-soluble dyes which were made on different animals. Although the fate of these compounds in the body was the main object of the investigation, observations were also made on toxicity. The following compounds were employed, the commercial names most commonly used and their numbers in Schultz's tables are given at the right.

Benzeneazo-β-naphthylamine	Yellow A.B.**
Tolueneazo-β-naphthylamine	Yellow O.B.**
Benzeneazobenzeneazo-β-naphthol	Sudan III 223*
Benzeneazo-β-naphthol	Sudan I 36*
Benzeneazodimethylaniline	Butter Yellow 32*
Benzeneazophenol	Oil Yellow.
Benzeneazoresorcinol	Sudan G 35*
Aminoazobenzene	Spirit Yellow 31*

* Schultz, Farbstofftabellen, Berlin, 1914.

** Heller and Mertz.

Rabbits were mainly used as subjects of the experiments but observations with some dyes were also made on a few cats and on rats.

Methods.

The dyes were administered subcutaneously, intraperitoneally, intravenously, and by mouth. For subcutaneous and intraperitoneal injections they were dissolved in cottonseed oil. In a few experiments at the beginning of the research, liquid gelatin or starch suspensions were also employed for this purpose in order to avoid a possible lipuria. Intravenous injections were given in 1 per cent sodium hydroxide or in emulsions made up with 5 per cent acacia, cottonseed oil, and sodium carbonate, the amount of dye in both cases being 0.25 per cent. All the dyes employed in these experiments were carefully purified before using since it had been shown by some investigators (10) that impurities caused a marked increase in their toxicity. In feeding experiments which were carried out on rats the dye

was incorporated with the food. Inquiries were also conducted to determine the effect of renal disturbance on the elimination of fat-soluble dyes in the urine. Zinc salts were mixed with the food and fed to rats with the dyes. Zinc malate was also introduced intravenously into rabbits, while the dye was given subcutaneously, a short space of time intervening between the two operations. Experiments were also made on the effect of oil of chenopodium on the elimination of dyes. The presence of dye in the urine and bile was determined by inspection and by dyeing wool.

I. The Elimination of Dyes in the Urine.

Whatever the mode of administration employed in our experiments, the presence of the different dyes in the urine (as well as in the bile) could be observed in every case in normal animals.

Their appearance in the urine could be observed within ½ to 2 hours after they were injected subcutaneously. Elimination usually continued several days and sometimes extended over several weeks even when only moderate doses were administered. Thus after the administration of 0.25 gm. per kilo of benzene- or tolueneazo-β-naphthylamine the dyes were present in the urine for 16 to 17 days. When 0.7 and 0.77 gm. per kilo of aminoazobenzene were given to two rabbits respectively the dye was still present in the urine at the end of 20 days. After injecting 0.6 gm. per kilo of benzeneazodimethylamine the urine was free from dye only at the end of 22 days. In experiments with benzeneazoresorcinol dye was still present 8 to 13 days after doses of 0.2 to 0.3 gm. per kilo were given. When 0.5 and 0.65 gm. of it per kilo were injected into two rabbits dye could be observed in the urine for 60 days. The rate of elimination by the kidney was much faster after intravenous injections. In most experiments the dyes appeared in the urine within 9 to 36 minutes. In some cases their appearance was delayed much longer. In one experiment it was first noticed in the urine after 80 minutes. The size of the dose seemed to have a marked influence on the speed of elimination, as was found in experiments with benzeneazoresorcinol. When about 100 mg. of the dye per kilo were given elimination lasted 1 to 3 days, while doses of 25 to 36 mg. per

kilo disappeared from the urine at the end of 2 to 6 hours. In two experiments, however, with 6 and 8 mg. per kilo elimination lasted 3 to 4 hours. It may be remarked that the medium in which the dye was administered was without any effect on the rate of elimination, the speed being the same whether given in the form of an emulsion or dissolved in sodium hydroxide.

That the different dyes employed (in these experiments) are also eliminated in the urine when they are given by mouth was shown in a number of experiments. Two cats received 100 mg. per kilo of benzeneazoresorcinol with their food. The next morning, that is, in about 17 hours, the urine was colored with dye. Elimination ceased in 24 to 48 hours. Similar results were obtained with other fat-soluble dyes that were fed to different animals. Rats that had been receiving daily with their food 60 to 70 mg. of benzeneazo-β-naphthylamine or 120 to 140 mg. per kilo of body weight of the toluene derivative eliminated these compounds in the urine; this was also observed in two rabbits that were given 1 gm. per kilo of these dyes by mouth. Elimination in the urine was likewise observed after intraperitoneal injections of these dyes when suspended in gelatin or starch as well as when dissolved in cottonseed oil. As tests for the presence of fat in the urine after the administration of the dyes were invariably negative, the observation that these substances are eliminated by the kidney suggested the advisability of ascertaining in what form they were present in the urine.

It was shown long ago by Schmiedeberg (11) that aniline given to animals is partly eliminated as glucuronate and partly as sulfate. It has also been established that various aniline derivatives undergo similar changes in the body, as shown in the case of antipyrin, acetanilide, and acetophenetidine. According to Gautrelet and Gravellat (12), marine blue is eliminated by the kidney in combination with sulfuric acid.

Conjugation with sulfuric or glucuronic acid was naturally thought of as the mechanism by which the various dyes which we have studied might be transformed into water-soluble compounds. This suggestion was tested in two dyes, benzeneazoresorcinol and benzeneazophenol, the following procedure being adopted.

The Separation of the Glucuronate of Benzeneazoresorcinol.

The urine of rabbits which had received the dye was treated with immiscible organic solvents such as amyl alcohol, ethyl acetate, benzene, ether, toluene, and chloroform, but all the attempts to extract it proved unsuccessful. Other procedures for the isolation of the substance were therefore resorted to. It was found that on treating the urine with dilute hydrochloric acid the dye could be precipitated. Care must be taken to avoid an excess of the acid as the precipitate may be redissolved if this precaution is not observed. After stirring thoroughly and cooling with ice, the mixture was allowed to stand several hours and then filtered by suction. The filtrate was still dark colored although no further precipitation could be obtained with acid. The brownish red precipitate was washed with cold water a few times and then crystallized from dilute alcohol. The substance crystallized in yellow to pale brown needle-shaped prisms melting at 189–190°C. with decomposition, the yellow substance changing to a red color at 130–140°C. without undergoing any other apparent transformation.

The nitrogen determinations were made by the modified Kjeldahl method of Phelps and Daudt (13). The substance was dried at 115°C. for 6 hours. The calculated amount of nitrogen in glucuronate of benzeneazoresorcinol, $C_{18}H_{20}N_2O_9$, was 6.86 per cent; found, 6.85 per cent.

Reactions of Glucuronate of Benzeneazoresorcinol.—The substance dyed wool yellow, gave no test for sulfur or halogen, and did not respond to the resorcinol test. On reduction in aqueous or alcoholic solution with stannous chloride the yellow color disappeared and did not return when exposed to the air, even when treated with oxidizing agents.

Hydrolysis.—0.5 gm. of the glucuronate was suspended in 100 cc. of water to which 2.5 cc. of HCl (sp. gr. 1.19) were added. Hydrolysis was effected by boiling the mixture until the solution became red. When boiled for 1 hour a red substance separated out on cooling. It was made slightly alkaline with sodium hydroxide and warmed on the steam bath for 1.5 hours, the clear red solution cooled, and then neutralized with acetic acid. A red precipitate was obtained which was filtered off. This substance was insoluble in water and crystallized from dilute alcohol in

beautiful acicular crystals which melted at 165°C. to a clear oil. When mixed with benzeneazoresorcinol (melting point 167–8°C. to an oil) the mixture also melted at 167–8° C. to a clear oil.

Furfurol-HCl Distillation.—0.5 gm. of the substance was suspended in HCl (sp. gr. 1.06) and distilled according to the method described by Abderhalden (14). The distillate gave a strong test with aniline acetate and on treatment with phloroglucinol the solution turned bright yellow, then green, and finally became turbid with a greenish black precipitate of the furfurol-phloroglucid.

Naphthoresorcinol Reaction.—The conjugated product was boiled in 10 cc. of water containing a few drops of concentrated HCl, cooled, and treated with an equal volume of concentrated HCl. (The substance would not respond to the test without this previous hydrolysis.) To this were added 2 cc. of an alcoholic solution of naphthoresorcinol and the mixture was boiled for 1 minute, allowed to cool, and extracted with ether. The ether took on a violet color and showed an absorption band at D.

The Separation of the Glucuronate of Benzeneazophenol.

The urine containing this substance behaved towards immiscible extracting solvents in a manner similar to the one previously described. The 24 and 48 hour urines of rabbits which had received benzeneazophenol were treated with concentrated HCl until precipitation was complete. The mixture was cooled with ice and the yellow precipitate filtered off by suction. This product was crystallized from dilute alcohol from which it separated in yellow rectangular plates or, sometimes, acicular prisms melting at 164–5°C. with decomposition.

The substance was dried at 110°C. for 4 hours. The amount of nitrogen calculated in benzeneazophenolglucuronate ($C_{18}H_{20}N_2O_8$) was 7.14 per cent; found, 6.85 and 6.76 per cent.

Reactions of the Glucuronate of Benzeneazophenol.—The substance dyed wool a light yellow, gave no test for sulfur or halogen, and did not respond to the Millon test. It was reduced by tin chloride in aqueous or alcoholic solution to a colorless solution but the color did not return on oxidation.

Hydrolysis.—0.15 gm. of the glucuronate was suspended in 25 cc. of water and treated with 0.6 cc. of concentrated HCl. The mixture was boiled gently under a reflux condenser, whereupon the substance gradually went into a light red solution with a floating dark red oil. The hydrolysis was continued for 4 hours and on cooling, yellow prismatic crystals separated. This substance was filtered off and washed with cold water. On crystallization from dilute alcohol peculiar hairy crystals were deposited which melted at 154–5°C. to a dark red oil. These crystals gave a Millon test and on mixing with some benzeneazophenol, which melts at 154–5°C. to an oil, the mixture also melted at the same temperature.

Furfurol-HCl Distillation.—65 mg. of the glucuronate were suspended in HCl (sp. gr. 1.06) and distilled as previously described. The distillate gave a strong Schiff test with aniline acetate and also a characteristic furfurol reaction with phloroglucinol.

Naphthoresorcinol Test.—This test was carried out exactly as described under the glucuronate of benzeneazoresorcinol. A positive reaction was obtained and the violet-colored ether showed an absorption band in the yellow at D.

On account of the color imparted to a solution of these glucuronates and the low specific rotation of glucuronic acid, no very satisfactory results could be obtained as regards optical activity. 1 per cent solution of the phenol derivative in 50 per cent alcohol showed a rotation of $-3°$ in a 0.5 dm. tube. After hydrolysis with dilute sulfuric acid this rotation was decreased to 0° but because of the energetic treatment which is necessary for the hydrolysis of this product, the glucuronic acid was probably decomposed and no positive rotation of the free acid could be detected. In another experiment where less material was utilized, the hydrolysis changed the rotation from -0.3 to $+0.1°$. However, these readings in dark colored fields are within the limits of experimental error. The synthesis with glucuronic acid, as established in the case of benzeneazophenol and benzeneazoresorcinol, indicates that the behavior of these substances in the body is similar to that of other members of the aromatic series. It may be inferred, therefore, that the other fat-soluble dyes whose elimination in the urine was observed had undergone a similar change, those not possessing a hydroxyl group first being oxidized to hydroxy derivatives.

II. The Elimination of Dyes in Diseases of the Kidney.

In a number of experiments on rabbits that received intravenously 10 mg. of zinc per kilo in the form of malate, marked albuminuria was present, severe lesion of the kidney being found on microscopic examination. The elimination of dyes which were given subcutaneously was very slight at first. In about 21 to 24 hours they completely disappeared from the urine.

As mentioned above, the elimination of fat-soluble dyes was observed in rats when these substances were incorporated with the lard that formed one of the ingredients of their diet. In two of these experiments zinc was given with the food, 40 gm. of which contained 60 mg. in the form of the acetate or of the sulfate. The urine examined 18 hours later failed to show the presence of the dye, thus indicating that renal function may be impaired even when the metal is given with the food.

Evidence of decreased permeability of the kidney was also present after the subcutaneous injection of oil of chenopodium, but the results were not constant, elimination being inhibited in only eight out of fourteen rabbits. Almost complete suppression of elimination was observed in one experiment in which the dyes were injected several days previous to the administration of oil of chenopodium. In two others, in which it was given immediately before the oil of chenopodium, the same results were obtained, none being eliminated during the first 24 hours, while the urine obtained on the following day was but slightly colored. In a third series of experiments, typical protocols of which are given below, the evidence of decreased permeability of the kidney after the administration of fat-soluble dyes was even more satisfactory.

Rabbit 1,671. Belgian Male. Weight 1,395 Gm. Diet, Oats.

Jan. 18. Subcutaneous injection of 0.75 gm. of benzeneazodimethylaniline dissolved in 15 cc. of cottonseed oil.

Jan. 19, 9 a.m. Urine collected. Contained considerable amounts of dye. 1 p.m. 0.55 cc. oil of chenopodium given by mouth in 15 cc. of cocoanut oil.

Jan. 20, 9 a.m. Rabbit found dead. The urine which passed since the oil of chenopodium was fed did not contain any dye. This rabbit had albuminuria for the last 6 days.

Rabbit 1,694. Black Male. Weight 1,930 Gm. Diet, Oats.

Jan. 21. Subcutaneous injection of 0.5 gm. of benzeneazophenol in 10 cc. of bleached cottonseed oil.

Jan. 22, 9 a.m. Urine collected. Contained a considerable amount of dye. 2.47 p.m. 0.8 cc. of oil of chenopodium in cocoanut oil given by mouth. Elimination of dye continued unchanged for the next 3 days, but the elimination was permanently suppressed after this.

Rabbit 1,665. Belgian Female. Weight 1,400 Gm. Diet, Oats.

Jan. 7, 11.10 a.m. Subcutaneous injection of 1 gm. of benzeneazophenol in bleached cottonseed oil. 4.10 p.m. Urine obtained from bladder showed presence of dye.

Jan. 11. Dye still eliminated in urine, 0.56 cc. of oil of chenopodium in 15 cc. of cocoanut oil given by mouth.

Jan. 12. Urine showed the presence of slight amounts of dye.

Jan. 13. Urine did not contain any dye. An examination of the urine daily for several days failed to indicate the presence of dye.

The experiment was repeated on another rabbit with the same result. In both cases albumin was found in the urine on the 3rd day after the injection of chenopodium and persisted for several weeks. In some rabbits, however, the elimination of dye continued uninterruptedly after oil of chenopodium was given although albumin was present in the urine. Permeability of the kidney for the dyes studied may be independent, therefore, of albuminuria which is frequently physiological in the rabbit (15). The failure of the dye to pass out in the urine could, nevertheless, be attributed to renal disturbance. It has been shown by Salant and Livingston (16), that oil of chenopodium produced a marked fall of blood pressure with a pronounced decrease in the volume of the kidney. Impaired function due to deficient nutrition caused by the lessened blood supply might thus interfere with the eliminating power of the kidney. This would seem to be highly probable in view of the experiments of Litten (17), who observed different degrees of injury after clamping the renal artery for $1\frac{1}{2}$ to 2 hours. On the other hand, abolition of the synthetic function of the liver might be regarded as the cause of the absence of the dye from the urine in poisoning with oil of chenopodium. That this was not the case appeared from experiments which were carried out

on rabbits with hydrazine, which is a hepatic poison. Oil-soluble dyes given to such animals were eliminated in the urine as was observed in the case of normal animals.

III. The Elimination of Dyes in the Bile.

Abel and Rowntree (18) observed that the oil-soluble tetrachlorophenolphthalein is eliminated in the bile but not in the urine. The experiments of Mendel and Daniels (9) with a number of fat-soluble dyes showed that the bile was the sole path of elimination for all those compounds which they examined. As shown in the preceding pages this view cannot be supported in the light of our observations on the subject. Their statement that these dyes may be eliminated by the liver, however, was fully corroborated by us in the present investigation. Examination of the bile, made after a proper interval following the administration of the different dyes which we have studied, showed that the liver played an important part in the elimination of these compounds. Bile removed from the gall bladder of a rabbit 14 days after the subcutaneous injection of toluneazo-β-naphthylamine contained a small amount of the dye. Evidence of elimination by this path was also obtained in experiments with benzeneazo-β-naphthol. The bile of three rabbits which received 0.5 to 0.6 gm. per kilo subcutaneously was still deeply colored at the end of 12, 13, and 18 days. The results were more striking after intravenous injections, the bile being obtained from a temporary fistula. In experiments on three rabbits which received 0.022, 0.029, and 0.043 gm. per kilo intravenously, the color was noticed in the bile in 16, 17, and 18 minutes respectively. Similar results were obtained in experiments with several other dyes. The period of elimination was found to be relatively short. After the intravenous injection of 0.130, 0.140, and 0.160 gm. of benzeneazoresorcinol per kilo, no dye could be observed in the bile at the end of 4 hours. It may be remarked that the period of elimination when a dose of 0.020 gm. per kilo of benzeneazophenol was injected intravenously was $3\frac{1}{4}$ hours. The probable significance of this will be discussed later. It is interesting to observe that elimination in the urine continued for some time after the dyes disappeared from the bile. After the subcutaneous injection of benzeneazoresorcinol

the bile in two experiments was free from dye at the end of 8 and 13 days but it was still present in the urine. This was also noticed after intravenous injections. A typical illustration of this is presented in the following experiment.

Rabbit 2,023.

9.45 a.m. Ether administered, cannula inserted in bile duct, and wound closed.
10.10. Bile light green.
10.21. 5 cc. of benzeneazoresorcinol in 1 per cent sodium hydroxide, injected into ear vein in 6 minutes.
10.36. Dye appeared in urine.
11.10. Urine obtained from bladder normal in appearance.
11.25. Urine very faintly tinged with dye.
11.40. Presence of dye in urine distinct.
1.30 p.m. 13 cc. of reddish brown bile collected. Bile light colored and became normal in appearance in about 1 hour.
4.15. 5 cc. of bile, yellowish green.
The following day, 9 a.m., 23 cc. of olive-green bile collected.
12.20 p.m. Urine obtained from bladder still colored with dye. Rabbit chloroformed.

The observation in this, as in several other experiments, that the dye continues to pass out in the urine after its disappearance from the bile is very suggestive, as it points to the kidney and not to the liver as being the chief organ of excretion for these compounds. This is particularly interesting in view of the results of Mendel and Daniels (9) who, it will be recalled, maintained that the fat-soluble dyes are eliminated entirely by the liver, their presence in the urine being observed only when lipuria existed. The decreased permeability of the kidney which we noticed in zinc nephritis suggests that a similar process might have been operative in the experiments of these investigators. Our observations on the inhibitory effect of oil of chenopodium on the elimination of dyes make this supposition highly probable for there was no evidence of renal injury caused by the oil, which indicates that the mechanism for the elimination of these substances might be deranged although no abnormality was observed in the appearance of the kidney or of the urine secreted. The discrepancy between the results of Mendel and Daniels and those which we obtained may thus be explained. It is entirely possible that the animals they used in their experiments were not normal.

IV. Are Dyes Eliminated into the Gastro-Intestinal Canal?

Observations have also been made on the elimination of fat-soluble dyes into the gastro-intestinal canal. In one experiment in which 0.29 gm. of benzeneazo-β-naphthol per kilo was given intravenously to a rabbit, the contents of the lower portions of the jejunum and of the ileum were stained but no evidence of the presence of dye in the rest of the gastro-intestinal canal could be obtained in this case. The experiment was repeated on another rabbit but no dye could be seen in any part of the gastro-intestinal canal. Tests with several other fat-soluble dyes used in this investigation likewise proved negative. The presence of the dye in the intestine might, therefore, be due to abnormal conditions. The recent report of Lewis (19) on trypan red is suggestive. After the intravenous injection of this substance into rabbits he observed large amounts of the dye in the aqueous humor when the eye was congested, but this fluid was free from color when the eye was normal.

V. The Distribution of Fat-Soluble Dyes in the Body.

Observations on this subject have been made by several investigators. Mann (8) claimed that animals fed oils colored with benzeneazobenzeneazo-β-naphthol show only staining of the adipose tissue. S. H. and S. P. Gage (7) failed to observe staining of the nerve fibers of chicks developed from eggs stained with benzeneazobenzeneazo-β-naphthol, but they reported that the adipose tissues were stained with the dye. Mendel and Daniels (9), who experimented with a number of fat-soluble dyes on different animals found that, with a few exceptions, the adipose tissue and bone marrow were stained. This was rarely the case with the muscles while the nervous system and kidneys appeared to be consistently free from dyes. Their presence in the liver could be observed in some cases but in the greater proportion of experiments no staining of this organ could be noticed after the administration of any of the coloring substances.

Our studies on the distribution of the different dyes in the body were made on rabbits and on cats. We found that the adipose tissues were always colored with the dye, no matter how introduced into the body, the color persisting even after its elimination

in the bile and urine had ceased. Staining of the other tissues was seldom noticed. In a few instances only could any dye be seen in the nervous system. A cat which had fasted 6 days received intraperitoneally 2 gm. of benzeneazo-β-naphthylamine per kilo, suspended in starch solution. The urine was colored until the death of the animal which occurred 45 hours after the dye was administered. On autopsy the dye could be seen very distinctly in the brain, nerves, voluntary muscles, subcutaneous tissues, and in the fat. It may be observed that a large amount of dye, apparently unchanged, was still present in the peritoneal cavity. A second experiment with this dye was repeated on a well fed cat, 1.3 gm. per kilo suspended in starch being administered intraperitoneally. The cat died 45 hours later. The subcutaneous tissues and fat of the body were likewise colored in this case; neither the brain nor spinal cord showed the presence of dye but the nerves were stained. The same results were obtained with tolueneazo-β-naphthylamine dye, which was also administered intraperitoneally to a well fed cat, a dose of 0.88 gm. per kilo suspended in starch being given. The duration of life in this case was 9 days. The urine contained the dye during the life of the animal. A dose of 1.1 gm. per kilo was given to another cat in the same way. The duration of life was nearly 6 days. Slight coloration of the subcutaneous tissues was observed but the nervous system was free from dye.

That the duration of life might be an important factor in the persistence of the various dyes in the tissues was shown in the following experiments in which the substance was introduced directly into the circulation. In two rabbits in which benzeneazoresorcinol, 0.1 gm. per kilo, was given intravenously it was present in the subcutaneous tissues, fat, brain, and nerves, in one of the rabbits which lived a little over 4 hours, while in the other, which lived 18 hours, the subcutaneous tissues and fat alone were colored. It may be remarked that in several experiments with different dyes, the nervous system was free from dye at the end of 4 to 6 hours after the intravenous administration. In one experiment on a rabbit 0.125 gm. per kilo of benzeneazo-β-naphthylamine was administered intravenously in the form of an emulsion. The rabbit lived 3½ hours. On autopsy the dye was found in the fat, subcutaneous tissue, brain, nerves, medulla of

the kidney, muscles, cartilage, and lungs. In similar experiments in which the duration of life was longer, the nervous system usually appeared normal in color. In a few cases, the nerves alone were stained. It might be added that the distribution of the dye was not restricted to fatty tissues and the nervous system. The femur was found markedly colored in one experiment after the intravenous injection of benzeneazoresorcinol; the bone marrow, however, was unstained in this case. The results concerning distribution may be briefly summarized by stating that the subcutaneous tissues and fat are generally colored after the administration of fat-soluble dyes. The brain may also contain dye occasionally, but the nerves are apt to be stained perhaps a little more frequently than the central nervous system.

We also made observations on the disappearance of dye from the blood. The tests were made with benzeneazoresorcinol only, which was given intravenously. In three experiments in which 25 and 67 mg. of the dye per kilo were introduced, it was still present in the serum after $9\frac{1}{2}$ to 15 hours. This is especially interesting since, as it will be recalled, elimination of the dyes we examined ceased in the bile and in the urine in 4 to 5 hours after their administration. It seems quite probable, therefore, that the elimination of these bodies occurs only as long as there are excessive amounts in the circulation. It may also be pointed out in this connection that some of the tissues of the body were stained when the bile and the urine no longer showed the presence of dyestuff. This observation that fat-soluble dyes may be present in the blood and other tissues in visible form after they can no longer be seen in the excretions might be due to fatigue of the excretory mechanisms for these compounds which, as we have seen, seem to be exceedingly delicate and unstable. That a firm combination might be formed with lipoids in the tissues from which the dye is either separated with difficulty or is transformed into a leuko compound and thus escapes detection, also appears to be a plausible explanation.

VI. Experiments on Toxicity.

Although the present studies were mainly confined to inquiries on the fate of fat-soluble dyes in the body, observations were also made on the toxicity of these compounds. It might be

stated in advance that none of the dyes examined were found to be very poisonous to the animals on which they had been tested. Thus, rabbits which received 1 to 2 gm. of benzeneazo-β-naphthylamine or of the toluene homologue (see Tables I and II) dissolved in oil and injected subcutaneously or intraperitoneally caused death in 4 to 9 days. A dose of 0.25 gm. per kilo was borne without manifestation of any untoward effects. It is interesting to observe that a dose of 1 gm. per kilo in oil given by mouth was more toxic than when given subcutaneously, the duration of life in one rabbit being $2\frac{3}{4}$ days, in another rabbit about 40 hours. As already mentioned, feeding experiments in rats were also carried out with these dyes. About 60 to 70 mg. per kilo of benzeneazo-β-naphthylamine fed daily with their food for $4\frac{1}{2}$ months and about twice these amounts of tolueneazo-β-naphthylamine administered in the same way for $6\frac{1}{2}$ months failed to show any poisonous action. Benzeneazoresorcinol (Table IV) given subcutaneously in amounts of 0.5 to 1.0 gm. per kilo likewise failed to induce any visible effect. A large number of experiments by intravenous injection were also made with this dye. 100 to 160 mg. per kilo dissolved in 1 per cent NaOH failed to produce any symptoms. When administered in the form of an emulsion, 100 mg. per kilo proved to be fatal to one rabbit in $4\frac{1}{3}$ hours and to another in 18 hours. Two other rabbits which received 86 and 94 mg. per kilo survived. The administration of larger doses could not be carried out satisfactorily on account of the lack of a suitable solvent. Of the other members of the group (Table VI), benzeneazo-β-naphthol, is perhaps more toxic, as rabbits that received 0.64 to 0.55 gm. per kilo subcutaneously died in 2 to 18 days, loss of appetite being the only symptom noticed. That this dye was toxic was claimed also by Weyl (10), who fed it to a dog. The duration of life of rabbits that received 1.7 gm. per kilo of benzeneazobenzeneazo-β-naphthol (Table III) subcutaneously and intraperitoneally was 1 to 3 weeks, which makes it extremely doubtful whether death was due to the dye. Mendel and Daniels (9) state that large doses fed to cats were harmless provided the dye was pure. Benzeneazobenzeneazo-β-naphthol is therefore also much less active than benzeneazo-β-naphthylamine or the toluene compound. After 0.7 and 0.77 gm. of benzeneazo-

phenol, loss of appetite was the only symptom observed. With the exception of one case (see asterisks, Table V) the rabbits survived. Its activity is therefore less than that of benzeneazo-β-naphthol. Benzeneazodimethylaniline dissolved in oil and given subcutaneously in doses of 0.53 to 0.6 gm. per kilo likewise failed to produce any symptoms except loss of appetite, which was observed in one rabbit. According to Weyl (10) this dye is not toxic to rabbits but Chlopin's (20) experiments on dogs which received 0.3 to 0.5 gm. per kilo by mouth, show that the dye may cause vomiting, diarrhea, and renal irritation. When such doses were repeated by him in 4 to 9 days he noticed severe symptoms of renal irritation and death.

Although the data on toxicity reported in the present investigation are of a preliminary character and are therefore inadequate, they nevertheless indicate important differences in the behavior of some of the dyes. As is shown in experiments with benzeneazobenzeneazo-β-naphthol, the toxicity of the naphthalene series may be decreased by the presence of another azo group, but even these compounds are more toxic than the phenol and resorcinol dyes. It may be remarked in this connection that Weyl (10), experimenting with a number of diazo colors, pronounced them non-poisonous.

SUMMARY.

1. Oil-soluble and water-insoluble dyes administered to different animals were eliminated in the urine and in the bile.

2. Elimination in the urine was usually inhibited in poisoning with zinc or oil of chenopodium.

3. Two of the compounds of benzeneazophenol and benzeneazoresorcinol, which were isolated from the urine of rabbits, proved to be conjugated with glucuronic acid.

4. Most of the dyes were deposited in the adipose tissues; staining of the nervous tissue, the kidney, and muscle was also observed in some experiments.

5. 10 to 15 hours after intravenous injection of 25 mg. per kilo of benzeneazoresorcinol, the dye was still present in the blood.

6. The toxicity of the different dyes was not pronounced even when larger doses were administered.

Experiments with Benzeneazo-β-naphthylamine (*Table I*).—Ten rabbits and two cats were used. After the injection no symptoms were observed except in the four cases noted. The duration of elimination was until death except in the four cases noted.

In the tables S stands for subcutaneous, P for intraperitoneal, and V for intravenous. "Tissue" refers to subcutaneous tissue.

TABLE I.

Animal.		Dye administered.			Elimination after:	Duration of life.	Remarks.
Weight.	Sex.	Per kg.	Mixture.	Injection.			
gm.		*gm.*			*hrs.*		
1,175	♀	1.7	In oil.	P	42	4¾ days.	No autopsy.
1,615	♀	1.2	"	"	42	6¾ "	Bile fistula. Tissue and fat not stained. Liver normal. Kidney congested and brownish. No dye in bile after 6 days.
1,250	♀	3.2	Suspended in gelatin.	"	19	2⅔ "	Fat stained. Liver friable. Heart injected. Much fluid in peritoneal cavity. Bile duct highly stained.
1,015 (Cat.)	♀	2.0	Suspended in starch.	"		21 hrs.	Bile from bladder did not dye wool. Dye unabsorbed. Tissue, fat, brain, nerves, and muscle stained. Cat was fasted previously for 6 days. Loss of weight 22 per cent.
1,570 (Cat.)	♀	1.3	"	"	21	45 "	Cat was fed previously. Loss of appetite after injection. Tissue, fat, and nerves stained. Liver and kidney normal. Spinal cord and medulla white. Stomach contents not colored. Intestinal contents slightly colored. Brain not stained.
1,050	♂	2.0	In oil.	S	22	5 days.	Dye unabsorbed. Tissue slightly stained. Brain very slightly stained. Nerves and muscles not stained.

TABLE I—*Concluded.*

Animal.		Dye administered.			Elimination after:	Duration of life.	Remarks.
Weight.	Sex.	Per kg.	Mixture.	Injection.			
gm.		*gm.*			*hrs.*		
1,270	♀	0.25	In oil.	S	17	9 days after 2nd injection.	Duration of elimination, 17 days. Then 2nd injection, 1.0 gm. No autopsy.
1,165	♂	1.0	"	"	18	7¾ days.	Dye unabsorbed. Tissue not stained.
1,015	♂	1.0	Suspended in gelatin.	"	Faint, 17½	37 "	Elimination continued very faint till death. Animal chloroformed. Dye found encysted at site of injection. Tissue and muscle not stained. Kidney normal.
825	♀	1.0	In oil.	*Per os.*	17½	2¾ "	Loss of appetite. Mucosa of small intestine, stomach, tissue, and fat stained. Nerves and muscle not stained.
1,045	♀	0.125	Emulsified.	V	1½	3½ hrs.	Fed carrot diet, 250 gm. per day. Paresis in 1½ hrs. Tissue, fat, brain, nerves, and muscle stained. Kidney medulla stained. Cartilage, lungs, and peritoneal fluid also stained.
1,610	♀	0.062	"	"	1½		Very slight depression only symptom. Duration of elimination, 10 days. Animal discarded.

Experiments with Tolueneazo-β-naphthylamine (Table II).—Seven rabbits and two cats were used. After the injection no symptoms were noted except loss of appetite in the four cases noted. The duration of elimination was until death except in the one case noted.

TABLE II.

Animal.		Dye administered.			Elimination after:	Duration of life.	Remarks.
Weight.	Sex.	Per kg.	Mixture.	Injection.			
gm.		*gm.*			*hrs.*	*days*	
1,675	♀	1.2	In oil.	P	20	2	Elimination in bile, dark red after 24 hrs. Bile fistula. Tissue and fat stained. Brain cortex and muscle slightly stained.
1,030	♂	2.0	"	"	19½	4	No autopsy.
1,200	♀	3.2	Suspended in gelatin.	"	18½	4	" "
1,850 (Cat.)	♂	1.1	Suspended in starch.	"	21	5¾	No appetite. Cat was fasted previously for 6 days. 13 per cent loss. Dye unabsorbed. Tissue slightly stained. Kidney normal. Peritoneal lining stained inside and out.
2,250 (Cat.)	♀	0.88	"	"	45 (21?)	9	Cat was fed previously. No appetite. Dye unabsorbed. Brain not stained. Tissue, fat, and nerves stained. Peritoneal lining stained inside and out.
1,070	♀	1.9	In oil.	S	22	5	Dye unabsorbed. Tissue, nerves, and muscle not stained.
1,160	♀	0.25	"	"	17	3¾ after 2nd injection.	Duration of elimination, 16 days. Then 2nd injection, 1.0 gm. Dye unabsorbed. Nerves not stained. Kidney slightly congested.
1,100	♂	1.8	Suspended in gelatin.	"	Faint, 17½	4	No appetite. Dye unabsorbed. Liver mottled.
950	♀	1.0	In oil.	*Per os.*	17½	1¾	No appetite. Tissue not stained. Stomach mucosa and secum slightly stained. Small intestine contained a little unabsorbed dye.

Experiments with Benzeneazobenzenezao-β-naphthol (Table III).—Four rabbits were used. After the injection no symptoms were observed except loss of appetite in the first two cases. The duration of elimination was until death.

TABLE III.

Animal.		Dye administered.			Elimination after:	Duration of life.	Remarks.
Weight.	Sex.	Per kg.	Mixture.	Injection.			
gm.		*gm.*			*hrs.*	*days*	
1,200	♀	1.7	Suspended in gelatin.	P	17	7⅔	Some dye unabsorbed. Tissue and brain not stained. Kidney congested.
1,240	♀	1.7	"	S	Faint, 17	20	Some dye unabsorbed. Tissue slightly stained.
1,215	♂	1.7	In oil.	"	Faint, 22	14	Bile dark wine-colored. No dye in bile, large and small intestine, or stomach. Brain not stained. Kidney congested.
1,900	♀	0.026	Emulsified.	V	70 min.*	22 hrs.	Bile fistula. Elimination in bile began in 14 min. Colored at death. Very slow secretion of bile, 4 cc. in 5¼ hrs. Before injection, secreted 8 cc. in 1 hr.

* No urine obtainable sooner.

Experiments with Benzeneazoresorcinol (Table IV).—The data for nineteen rabbits are shown in the table.* The injection was intravenous in the first twelve cases, subcutaneous in the rest.

TABLE IV.

Animal.		Dye administered.		Elimination after:	Duration of:		Remarks.
Weight.	Sex.	Per kg.	Mixture.		Elimination.	Life.	
gm.		*gm.*		*min.*	*hrs.*		
1,610	♀	0.094	Emulsified.	10	23	72 hrs.	Injection in divided doses. 31 mg. per kg. injected at a dose. Animal chloroformed. Tissue and fat stained. Fatty degeneration of lumbar muscles. Nerves not stained.
1,735	♀	0.029	"	15	4½	6 "	Animal chloroformed. Tissue and fat stained. Urine in bladder normal. Nerves not stained.
		0.029	"	10	4–5		
1,530	♀	0.100	In NaOH.	9	±48		Discarded. Autopsy 9 days after injection. Tissue and fat slightly stained. Bile and urine normal.
1,700	♀	0.100	"	9	±24	72 "	Two injections in 15 min. Animal chloroformed. Tissue and fat stained. Femur stained inside and out. Bone marrow not stained.
1,910	♀	0.026	"		5–6		Discarded.
1,890	♀	0.026	"	15	3–4		"
1,730	♂	0.028	"	15	±5	4 days.	Animal chloroformed. Tissue and fat slightly stained. Blood in urine obscured accurate observation.
1,470	♂	0.013	"	25	3		Fasted 7 days before injection. Discarded.
1,790	♀	0.067	"			22 hrs.	Tissue stained. Serum stained 10 hrs. after injection.
1,970	♀	0.025	"			22 "	Bile light red-brown. Tissue and fat stained. Urine in bladder normal. Serum stained 9½ hrs. after injection.

* The total number of rabbits employed in experiments with benzeneazoresorcinol was thirty-four.

TABLE IV—*Concluded.*

Animal.		Dye administered.		Elimination after:	Duration of:		Remarks.
Weight.	Sex.	Per kg.	Mixture		Elimination.	Life.	
gm.		*gm.*		*min.*	*hrs.*		
1,985	♂	0.025	In NaOH.			20 hrs.	Animal chloroformed. Tissue stained. Fat slightly stained. Urine in bladder normal. Serum stained 15 hrs. after injection, though bile was normal in color.
2,330	♀	0.086	Emulsified.	10	±72	4 days.	Animal chloroformed. Kidney normal. Urine normal. Spleen very hard, brittle, and *yellow*. Tissue slightly stained. Fat, brain, and nerves not stained.
				hrs.	*days*		
2,030	♀	0.5	In oil.	3			Brain and nerves not stained. Dye in urine disappeared in 2 days. Treated with chenopodium. Dye unabsorbed.
1,300	♂	0.75	"	2			Treated with chenopodium. Dye unabsorbed.
1,950	♀	0.5	"	17	60		Survived and was discarded.
1,075	♀	1.0	"	17	60		" " " "
2,910	♀	0.28	"	½	8	8 "	Animal chloroformed. Fat slightly stained. Adrenals seemed stained, chyle cream-colored. Urine normal. Dye unabsorbed.
1,980	♂	0.2	In NaOH.	17	12	13 "	Bile fistula.
2,150	♀	0.23	"	1			Given hydrazine.

Experiments with Benzeneazophenol (Table V).—The data for four rabbits are shown in the table.* No symptoms were observed except loss of appetite in the first case.

TABLE V.

Animal.		Dye administered.		Elimination after:	Remarks.
Weight.	Sex.	Per kg.	Mixture.		
gm.		*gm.*		*hrs.*	
1,400	♀	0.7	In oil.	5	Oil of chenopodium given the day after dye. Elimination of dye inhibited in 72 hrs. Rabbit given 1.0 gm. of dye 4 weeks later. Lived 3 days.
1,960	♂	0.5	"	4	Oil of chenopodium given 4 days after dye. Elimination of dye inhibited in 24 hrs. Survived.
1,910	♀	0.7	"	3	Lived 5 days. Urine in bladder contained dye; no dye in bile.**
1,165	♀	0.43	In NaOH.	17½	Bile in gall bladder normal color.

* The total number of rabbits employed in experiments with benzeneazophenol was seven.

** See page 418.

Experiments with Benzeneazo-β-naphthol (Table VI).—Ten rabbits were used, all males except the first. The injection was subcutaneous in all but the last three cases, in which it was intravenous. Loss of appetite was the only symptom observed. The duration of elimination was until death unless otherwise noted.

TABLE VI.

Weight of animal.	Dye per kg.	Elimination after:	Duration of life.	Remarks.
gm.	*gm.*	*hrs.*		
1,325	0.75	21	9 days.	No elimination in urine after 4½ hrs. Oil of chenopodium on 6th day; elimination of dye markedly decreased. Some dye not absorbed. Tissue stained. Nerves and muscles not stained.
1,680	0.59	17	11 "	
1,570	0.64	1	48 hrs.	Dye present in bile, liver, and kidney. Fat stained. Absorption incomplete. Tissue not stained.
1,800	0.55	17	18 days.	Dye present in bile.
1,995	0.5	17	13 "	Bile fistula made 13 days after injection. Dye present in bile.
1,670	0.6	21	7 "	Fasted 5 days before injection. Some dye unabsorbed. Tissue and fat stained.
1,855	0.55	21	12 "	Fasted 5 days before injection. Some dye unabsorbed. Tissue and fat stained. Brain and nerves not stained. Dye present in bile.
1,740	0.029	¾	4½ hrs.	Bile fistula. 16 min. after injection, dye in bile but secretion of bile stopped.
2,270	0.022		19 "	Bile fistula. Dye present 16 min. after injection. Secretion of bile slow. Urine secreted over night contained small amount of dye.
1,150	0.043	1	21½ "	Bile fistula. Dye appeared 8 min. after injection. Bile secretion before injection, 5 cc. in 35 min.; after injection, 4 cc. in 5 hrs., and none after this.

BIBLIOGRAPHY.

1. Daddi, L., *Arch. ital. biol.*, 1896–97, xxvi, 143.
2. Biedermann, W., *Arch. ges. Physiol.*, 1898, lxxii, 105.
3. Sitowski, L., *Anz. Akad. Wissensch. Krakau*, 1905, 542.
4. Hofbauer, J., Grundzüge einer Biologie der menschlichen Plazenta mit besonderer Berücksichtigung der Fragen der fötalen Ernährung, Vienna, 1905.
5. Riddle, O., *J. Exp. Zool.*, 1910, viii, 163.
6. Gage, S. H., and Gage, S. P., *Science*, 1908, xxviii, 494.
7. Gage and Gage, *Anat. Rec.*, 1908, iii, 203.
8. Mann, G., Physiological Histology, Oxford, 1902, 306 and 307.
9. Mendel, L. B., and Daniels, A. L., *J. Biol. Chem.*, 1912, xiii, 71.
10. Weyl, T., Die Teerfarben, Berlin, 1889.
11. Schmiedeberg, O., *Arch. exp. Path. u. Pharm.*, 1878, viii, 1.
12. Gautrelet, G., and Gravellat, H., *Compt. rend. Soc. biol.*, 1907, lxii, 96.
13. Phelps and Daudt, paper to be published.
14. Abderhalden, E., *Handb. biochem. Arbeitsmethoden*, 1910, ii, 130.
15. Backman, E. L., *Compt. rend. Soc. biol.*, 1916, lxxix, 339.
16. Salant, W., and Livingston, A. E., *Am. J. Physiol.*, 1915, xxxviii, 67.
17. Litten, M., *Arch. path. Anat., u. Physiol.*, 1881, lxxxiii, 508.
18. Abel, J. J., and Rowntree, L. G., *J. Pharm. and Exp. Ther.*, 1909–10, i, 231.
19. Lewis, P. A., *J. Exp. Med.*, 1916, xxiii, 669.
20. Chlopin, cited after Hesse, B. C., *U. S. Dept. Agric., Bureau of Chemistry, Bull. 147*, 1912.

AN ELECTRICALLY HEATED VACUUM DESICCATOR.*

By T. BRAILSFORD ROBERTSON and CARL L. A. SCHMIDT.

(From the Department of Biochemistry and Pharmacology, Rudolph Spreckels Physiological Laboratory, University of California, and the Hearst Laboratory of Pathology and Bacteriology, University of California, Berkeley.)

(Received for publication, September 27, 1916.)

In preparing certain biochemical products it is frequently desirable to employ a desiccator of large capacity, capable of evacuation and of being heated and maintained at a predetermined temperature. The various forms of apparatus available on the market are either of very small capacity or else expensive and deficient in the range and adjustability of the temperatures employed. We accordingly have had constructed for our use the apparatus described below, which is of large capacity, readily accommodating a filter funnel 9 inches in diameter containing a 50 cm. filter paper. It maintains a vacuum for several hours and may be held with considerable precision at any desired temperature within a wide range. If desired, a vessel containing sulfuric acid or other desiccating reagent may be placed on the floor of the apparatus and false bottoms or shelves may be fixed at any desired height above the reagent. With the aid of this apparatus, employing certain obvious precautions, tethelin suspended in alcohol-ether mixture may be completely dried without discoloration at 36°C. within 6 hours, yet this substance is so exceedingly hygroscopic that a few minutes' exposure to the air of the room suffices to convert it into a deeply colored, sticky mass.[1]

The apparatus consists of a double walled circular copper ($\frac{1}{16}$ inch sheet copper) chamber, well insulated by asbestos and set in a monel metal shell. This chamber can be closed at the top

* Aided in part by a grant from the George Williams Hooper Foundation for Medical Research.

[1] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 409.

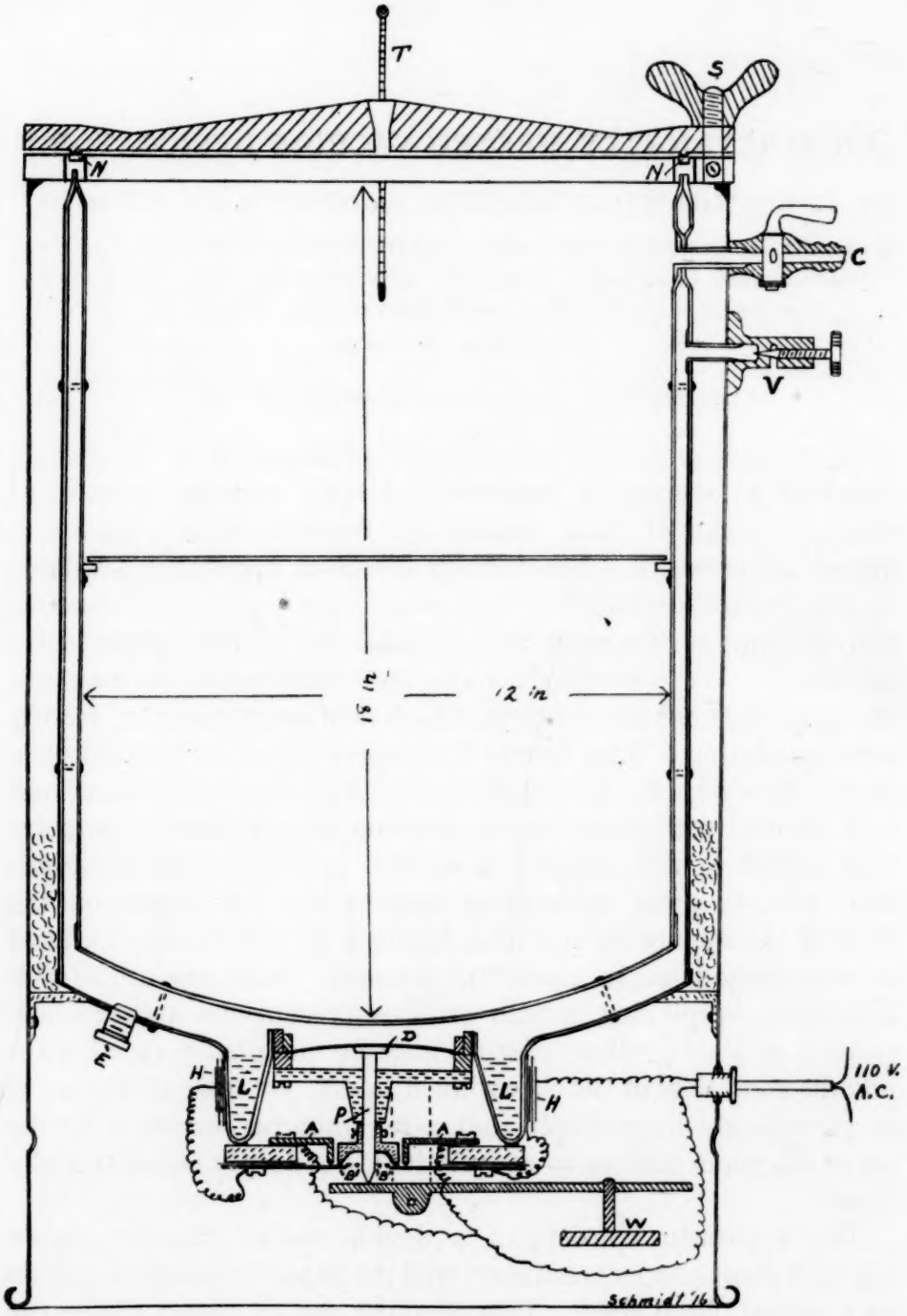


FIG. 1. An electrically heated vacuum desiccator. Scale $\frac{1}{5}$.

by a tight fitting cast aluminum lid which can be screwed down by means of three screws (S). A rubber gasket in the notched space (N) makes the compartment air-tight. The chamber is evacuated by attaching a vacuum pump to the cock (C) and the vacuum so obtained can readily be maintained for some time after the cock is closed. The desired temperature in the evacuated chamber is maintained by the heated vapor of dichloromethane (boiling point 40°C.) which fills the space between the two copper walls, heat being furnished by eight 25 watt nichrome heating elements, connected in parallel and placed around the pocket (L). Each heating element is in turn connected to a small platinum-tipped brass spring (B) which makes contact with a platinum point set on the ring (A). In this way one or more of the heating elements can be used as needed.

Temperature control is maintained by means of the sprung copper disc (D). When heat is applied to the liquid dichloromethane in (L), vapor is formed which will exert a pressure on the copper disc (D), tending to force it downward. This disc in turn presses against the pin (P) which when forced downward will cause the contact between (A) and (B) to be broken. In this way any or all of the heating elements (H) are switched in or off as needed to maintain a constant vapor pressure and temperature in the double walled space surrounding the chamber. By shifting the weight (W), tension on the disc (D) may be increased or decreased and the temperature in the chamber varied (within a certain range) at will. By selecting liquids having a boiling point higher than that of dichloromethane the temperature range may also be varied. Heating of the chamber by means of the vapor of a volatile liquid assures a uniform distribution of heat.

On using the apparatus for the first time the needle valve (V) is opened to allow air to escape and closed again when dichloromethane vapor begins to escape. This assures the space being filled solely with dichloromethane vapor. To fill or drain the liquid in (L) a drain plug (E) is provided. To insure rigidity and tightness, the walls are thoroughly braced and reinforced and all joints are silver-soldered.

The apparatus was constructed by Mr. A. J. Kercher of Berkeley and embodies several of his own inventions.

THE OXIDATION OF BRANCHED CHAIN FATTY ACIDS.

I. THE ACTION OF HYDROGEN PEROXIDE ON THE HOMOLOGUES OF ISOBUTYRIC ACID.

By P. A. LEVENE and C. H. ALLEN.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, September 25, 1916.)

It is noteworthy that while the majority of fatty acids occurring in nature belong to the normal series, those composed of more than twenty-two carbon atoms contain at least one tertiary carbon atom in their molecule.[1] The structure of these acids is entirely unknown. The problem of their structure naturally is identical with that of the location of the tertiary carbon atom in the molecule. There exist two methods by means of which the solution of the latter problem may be reached. One consists in the gradual transformation of a fatty acid into its lower homologue passing through the following phases:

$$RCH_2COOH \rightarrow RCHBrCOOH \rightarrow RCHOHCOOH \rightarrow RCOOH$$[2]

By continuing these operations on the lower homologues one may finally reach an acid of known structure, or one the structure of which can be made known by synthesis. This method has the advantage of generality and reliability; however, it has the disadvantage of requiring large quantities of material and much time for execution.

The second possible method is to be based on the properties of the tertiary carbon atom. In aliphatic hydrocarbons, alcohols, acids, etc., containing a tertiary carbon atom ($\equiv$ CH), this is found to be most susceptible to the action of oxidizing agents.

However, the experience gained in this laboratory on lignoceric and cerebronic acids was rather disappointing. Lignoceric acid

[1] See Meyer, V., and Jacobson, P., Lehrb. organ. Chem., Leipsic, 2nd edition, 1907, i, 501.

[2] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1914, xvi, 475.

was shown by Meyer, Brod, and Soyka[3] to contain a tertiary carbon atom. Levene and West[4] have substantiated this conclusion and have also demonstrated the presence of a tertiary carbon atom in cerebronic acid. Yet when lignoceric or cerebronic acid was heated for a considerable period of time in a permanganate solution one could find no evidence of the oxidation of the tertiary carbon atom.

On the other hand, there is no reason to doubt that under favorable conditions this oxidation may take place. The problem then, as it presented itself at this phase, was to establish conditions under which the tertiary carbon atom undergoes oxidation readily. The undertaking seemed all the more desirable since the conclusions regarding the properties of the methenyl group (≡ CH) of the earlier writers were based on a very limited number of observations. It was realized that the character of the radicles attached to the tertiary carbon atom may be of considerable influence on its stability. In the series of aliphatic acids the distance of the tertiary carbon atom from the carboxyl seemed particularly worthy of consideration. In view of this it was concluded to investigate the action of oxidizing agents on the higher homologues of dimethylacetic (isobutyric) acid, $(CH_3)_2CH \cdot COOH$. Unfortunately only the lower members of this series had been prepared by previous workers and our task fell into two parts, one the preparation of the higher homologues, and the other their oxidation.

Preparation of the Acids.

The preparation of the acids was based on the following set of reactions:

$$RCH_2OH \rightarrow RCH_2I \rightarrow RCH_2CH \cdot (COOC_2H_5)_2 \rightarrow RCH(COOH)_2 \rightarrow RCH_2COOH \rightarrow RCH_2COOC_2H_5 \rightarrow RCH_2CH_2OH$$

The set of reactions was then continued progressively to build up the higher homologues.

2-Methylpropyl alcohol (isobutyl alcohol) $(CH_3)_2CH \cdot CH_2OH$,

[3] Meyer, H., Brod, L., and Soyka, W., *Monatsh. Chem.*, 1913, xxxiv, 1113.

[4] Levene and West, *J. Biol. Chem.*, 1914, xviii, 477.

and its next higher homologue, 3-methylbutyl alcohol (isoamyl alcohol) $(CH_3)_2CHCH_2 . CH_2OH$, served as the starting material.

The first substance was obtained from Kahlbaum and from the Du Pont Chemical Works, and purified by fractional distillation. The second was prepared from the so called "amyl alcohol," following the procedure first suggested by Pasteur and later perfected by Marckwald.[5] The directions of the latter investigation were followed rather in principle than in detail. After some experience in handling the large volumes of liquids the yield was very satisfactory.

The iodides were prepared by distilling the alcohols with hydriodic acid. The procedure was essentially the same as recommended by Norris.[6] Only such slight modifications were introduced as suggested themselves by the larger quantities of material employed in course of this work. The details are given in the experimental part. The yields were never less satisfactory than those obtained by Norris, and were at times considerably better.

Reduction of the Esters to Alcohols.—For the reduction of esters into the corresponding alcohols the process of Bouveault and Blanc[7] has been essentially improved. In the original process an alcoholic solution of the ester is slowly added from a dropping funnel to six atoms of sodium divided in large pieces. The procedure was rather awkward and the efficiency of the sodium minimal. In the present work the sodium was suspended in toluene. The flask was provided with a mechanical stirrer. By heating the mixture in a metal bath with continuous stirring the sodium was reduced to small globules. When this stage was reached, alcohol and then the ester in alcoholic solution were allowed to flow from a dropping funnel into the emulsion of sodium and toluene. By continuous stirring the contact surface between sodium and ester was maintained at its maximum. The time of reaction is reduced from 6 hours by the original method to 1 hour by the modified method. The apparatus and the details of the procedure are described in the experimental part.

All the other steps in the preparation of the acids were those of

[5] Marckwald, W., *Ber. chem. Ges.*, 1901, xxxiv, 479; 1902, xxxv, 1595.

[6] Norris, J. F., *Am. Chem. J.*, 1907, xxxviii, 627. Norris, J. F., Watt, M., and Thomas, R., *J. Am. Chem. Soc.*, 1916, xxxviii, 1071.

[7] Bouveault, L., and Blanc, G., *Bull. Soc. chim.*, 1904, xxxi, 666.

the malonic ester synthesis, and were carried out in the conventional way.

In the course of this work the acids, esters, alcohols, iodides, and substituted malonic acids of eight homologues of isopropane were prepared. The isobutyl and isoamyl derivatives had for their starting materials the corresponding alcohols. The normal changes in physical properties with an increase in the number of carbon atoms in the chain were noted. The specific gravities of the alcohols increased progressively. The solubility in water of the dibasic acids decreased and their solubility in petroleum ether increased with an increase in the hydrocarbon part of the molecule. The melting points of the dibasic acids, the phenylurethanes of the alcohols, and the amides of the monobasic acids showed the customary irregularity. Table I shows the record of the constants of the new acids and of the intermediate products in the preparation of the acids.

TABLE I.

Physical Constants of Compounds with the Radicle $(CH_3)_2CH(CH_2)x$.*

No. of carbons in R.†	RCH_2OH b. p.	$C_6H_5NH.CO.OCH_2.R$ m. p.	RCH_2I b. p.	RCOOH b. p.	$RCOOC_2H_5$ b. p.	$RCONH_2$ m. p.	$RCH(COOC_2H_5)_2$ b. p.	$RCH(COOH)_2$ m. p.
4	130‡	56.6	148‡	174‡	134.3‡	127–129‡	240–242‡	93
5	153	48.0	173.2	199.3‡	160.4‡	120‡	137.0_{11}	86.5
6	170.5	82.5	195.2	216	182.7	103.5–104.0	155_{15}	100.0–100.3
7	188.5	81.0–81.4	100.0_{17}	126–127_{14} 232	200.3	114.0	171.8_{18}	89.5–90.0
8	206.0	66.4	120.0_{20}	140.5_{15} 248	220.5	106.5	182_{18}	92
9				155.6_{16}				
10				174.0–174.5_{25}				

* All data taken with standard thermometer and corrected.

† R = $(CH_3)_2CH(CH_2)x$.

‡ Taken from the literature and in every case where the compound was prepared, verified by this work.

Oxidations.

In selecting the oxidizing agent there was a choice of nitric acid, chromic acid, permanganate, or hydrogen peroxide. Every agent has advantages and disadvantages. On oxidation with nitric acid the reaction is complicated by the formation of nitro derivatives.[8] The oxidation with potassium permanganate may, according to Przewalsky,[9] give rise to isomerization. Chromic acid also has some disadvantages, particularly because the reagent adheres tenaciously to the products of oxidation. However, it is planned to test systematically the individual peculiarities of each of these agents. The present work was limited to experiments with hydrogen peroxide. It was selected because it presents the greatest convenience in manipulation.

From the work of Dakin[10] it is known that normal fatty acids undergo the so called β oxidation, the acid giving rise to a ketone having in its carbon chain one atom of carbon less than the original acid:

$$RCH_2CH_2COOH \rightarrow RCOCH_3$$

To a smaller degree they oxidize in the α-position with the formation of the next lower aldehyde:

$$RCH_2COOH \rightarrow RCHO$$

Regarding the action of hydrogen peroxide on the branched chain fatty acids the observations are limited to those of Dakin on isobutyric and isovaleric, and to those of Raper on isobutyric and on α-methylbutyric acids. In isovaleric acid the tertiary carbon atom happens to be situated in the β-position, which renders it doubly susceptible to the action of hydrogen peroxide. Raper,[11] reports besides the usual oxidation of the tertiary carbon atom, also a peculiar behavior of the α-methyl group. The present work does not deal with acids of this structure and hence this new type of oxidation will be omitted from the present discussion. However, the fact that the predominating product of

[8] Bredt, J., *Ber. chem. Ges.*, 1882, xv, 2324.

[9] Przewalsky, E., *J. prakt. Chem.*, 1913, lxxxviii, 495.

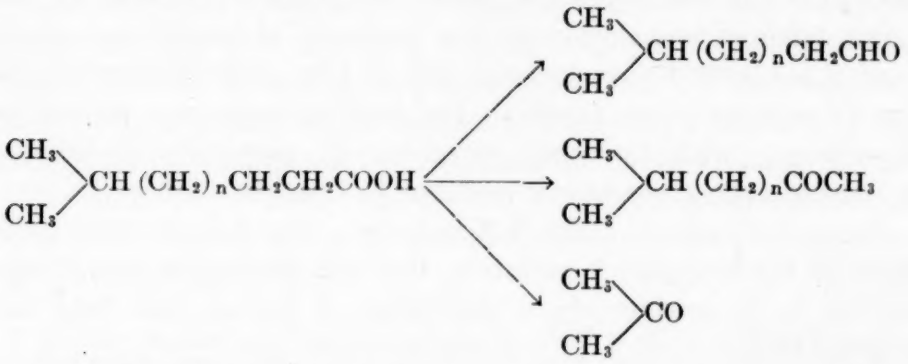
[10] Dakin, H. D., Oxidations and Reductions in the Animal Body, London, 1912.

[11] Raper, H. S., *Biochem. J.*, 1914, viii, 320.

oxidation of α-methylbutyric acid was methylethyl ketone goes to show that in this substance the tertiary carbon atom is the principal locus of oxidation.

In course of the present investigation the higher homologues of isobutyric acid (including 9-methyldecylic acid) were subjected to the action of hydrogen peroxide.

On the basis of facts which have been referred to in this discussion, the following products of oxidation were to be expected.



The principal aim of the work was to find the conditions which favor the third reaction above the other two, and furthermore to investigate the influence on this reaction of the distance of the tertiary carbon atom from the carboxyl group.

It was found that the reaction proceeded most favorably when the solution of the organic acid was slightly acid and when an amount of peroxide solution equivalent to three and one-half atoms of oxygen was employed for the reaction. Under such conditions all of the three reactions outlined above took place. However, acetone was the predominating product of reaction. Acetone was identified as its *p*-nitrophenylhydrazone, which showed the correct melting point and composition.

The presence of the higher ketones was demonstrated by an indirect method; the value of the mixed ketones was determined and from this was deducted the value of the acetone present in the same solution.

The aldehydes resulting from the oxidation in the α-position were identified as the corresponding acids.

The plan of the analytical work was essentially the same as employed by Dakin and by Raper, and is given in detail in the experimental part.

As regards the yields of acetone, there was noted a progressive fall with the increase of the distance between the tertiary carbon atom and the carboxyl group. A marked exception to the rule was observed in the case of isobutyric and isovaleric acids. Contrary to the general rule the yield of acetone was greater from the higher homologue. Undoubtedly this is occasioned by two factors. First, in isovaleric acid the acetone is formed by two types of oxidation; the β oxidation, and the oxidation of the tertiary carbon atom as such; on the other hand, in isobutyric acid the β oxidation leads to demethylation and hence proportionally diminishes the mass of the tertiary carbon atoms. The demethylation of isobutyric acid was demonstrated by Raper, who found propionic aldehyde among the products of oxidation of this acid. In the present work propionic acid was found directly among the products of oxidation.

Incidentally it may be mentioned that all attempts to isolate dicarboxylic acids from the non-volatile part of the oxidation product were futile. Furthermore, the acids extracted from this fraction on titration gave molecular weight values corresponding closely to the original acids. Hence it seems possible that the remainder of the molecule after the formation of acetone undergoes complete oxidation to carbon dioxide.

EXPERIMENTAL PART.

3-Methylbutyl Alcohol (*Isoamyl Alcohol*).—Constant boiling amyl alcohol having a rotation of $-0.75°$, indicating 12.7 per cent of the active alcohol, was mixed with an equal weight of concentrated sulfuric acid. The temperature during the mixing was kept below 40°C. The next day water and ice were added until the liquid had been diluted to double its volume. Solid barium hydroxide was then added with mechanical stirring until the liquid was neutral to Congo red. During this process also, the temperature was kept below 40°C. The precipitated barium sulfate was washed by decantation until all of the barium amyl sulfate had been removed. The wash liquors were repeatedly concentrated under diminished pressure and allowed to crystallize until the resulting crystals showed a greater rotation than $+0.20°$ in 10 per cent solution in a 2 dm. tube at room temperature. All mother liquors

and crystals having a rotation greater than this were rejected. As barium amyl sulfate of this purity makes about a 10 per cent solution in water at room temperature the rotation of the mother liquors and of the crystals under the conditions stated above are comparable.

About 25 per cent of the alcohol was recovered from the distillates of the wash liquors. The weight of the crude salt was approximately equal to the weight of the alcohol originally taken. The first yield of crystals from the wash liquors had a rotation of 0.05 to 0.08°. The barium amyl sulfate was recrystallized either from two and a half times its weight of water or from mother liquors of higher purity until it showed no rotation in 10 per cent solution in a 2 dm. tube. After this it was recrystallized once from water. All the mother liquors not used for recrystallizing were concentrated to crystallization.

The inactive salt was decomposed in hot concentrated solution with a moderate excess of strong sulfuric acid. The precipitated barium sulfate was washed by decantation and the wash liquors refluxed for 3 hours to hydrolyze the isoamyl sulfate. A layer of alcohol separated out. This was removed. The alcohol dissolved in the aqueous layer was recovered by distillation. The alcohol obtained in this manner contained as impurities, sulfuric acid and isoamyl sulfate. For purification two-thirds of the original volume were distilled off directly and the remainder prior to distillation was freed from impurities by washing with water.

The alcohol obtained in this way had a rotation of 0.04 to 0.08° in a 1 dm. tube at room temperature. The yield was from 10 to 15 per cent. 4 kilos of inactive alcohol were used in the course of the following work.

3-Methylbutyl Phenylurethane.—2 gm. of inactive alcohol and 2.7 gm. of phenylisocyanate were heated under a reflux for a few minutes. The liquid solidified on cooling. After four crystallizations from dilute alcohol the melting point showed no further change. Melting point 56.6°C. (corrected).

0.2006 gm. substance neutralized 9.57 cc. 0.1 N HCl (Kjeldahl).

	Calculated for $C_{12}H_{17}NO_2$:	Found:
N	6.76	6.68

3-Methylbutyl Iodide.—50 gm. lots of the inactive alcohol were slowly distilled with 224 cc. (three equivalents) of constant boiling hydriodic acid. The distillation was continued until the temperature reached 127°C. At this point all of the iodide had distilled over. A new portion of the alcohol and enough acid to make up the original volume were then added and the distillation was continued. The iodide was separated from the distillate, washed with water, decolorized with sodium thiosulfate, dried over sodium sulfate, and distilled. The low boiling portions were redistilled with hydriodic acid. The yield was 88 per cent of the theory. 750 gm. of the alcohol gave 1,485 gm. of the iodide.

5-Methylhexylic Acid.—3-Methylbutyl iodide was condensed in the customary manner with malonic ester in lots of 50 to 100 gm. The 3-methylbutylmalonic ester was washed with water and then saponified without further purification.

For saponification the wet ester was vigorously stirred with twice the theoretical amount of 50 per cent sodium hydroxide. The mass soon became hot and then solidified. The evaporation of the alcohol and water kept the temperature below 100° and additional cooling was not required even when lots of 800 gm. were saponified. The soap was extracted twice with acetone, then mixed with a small amount of water and acidified with concentrated hydrochloric acid with cooling. A large part of the 3-methylbutylmalonic acid separated out and later solidified. The sodium chloride was filtered off and extracted twice with ether, the mother liquors were extracted twice with ether in a separatory funnel and then for 4 hours in Thiele continuous extractors. From the latter extract about 10 per cent of the yield was obtained. For later preparations the acid dissolved in the mother liquors was recovered more conveniently but less economically by precipitating it from ammoniacal solution as the calcium salt. The ether solution of the acid was dried, the ether removed, and the syrup crystallized over sulfuric acid in a vacuum desiccator. The crystals were washed with petroleum ether. The constants found for this acid and its ethyl ester agreed with those reported by the earlier workers.[12] The acid melts without decom-

[12] Paal, C., and Hoffmann, T., *Ber. chem. Ges.*, 1890, xxiii, 1496. Frankland, E., and Duppa, P. F., *Ann. Chem.*, 1866, cxxxviii, 339. Fournier, H., *Bull. Soc. chim.*, 1909, i, 925. Nef, J. U., *Ann. Chem.*, 1901, cccxviii, 146. Grimshaw, H., *Ann. Chem.*, 1873, clxvi, 168. Wallach, O., *Ann. Chem.*, 1915, cdviii, 190.

position at 93°C. The yield was 83 per cent of the theory, calculated on the basis of the iodide. 1,300 gm. of 3-methylbutyl iodide gave 1,000 gm. of the dibasic acid. The acid crystallizes readily from benzene. It dissolves readily in water and is almost insoluble in petroleum ether.

The dibasic acid was decomposed in 100 gm. lots by heating in a distilling flask at 170–180° for half an hour. The temperature was then raised and the 5-methylhexylic acid distilled. 410 gm. of crude dibasic acid gave 263 gm. of the acid. The yield of 5-methylhexylic acid on the basis of the fraction boiling at 214–216° (corrected) was 85 per cent of the theory. The 5-methylhexylic acid was neutralized with sodium hydroxide and the solution distilled with steam to free the soap from impurities. 5-Methylhexylic acid has been prepared by several workers.[12] There is a lack of agreement between the boiling points reported by them, the variation ranging between 208 and 214–216°C. The boiling point of the substance described in this report was 216°C (corrected) at 762 mm. pressure.

0.1327 gm. substance gave 0.3130 gm. CO_2 and 0.1301 gm. H_2O.

	Calculated for $C_7H_{14}O_2$:	Found:
C	64.57	64.32
H	10.85	10.98

Ethyl 5-Methylhexylate.—The acid was esterified by refluxing for 3 hours with three equivalents of absolute alcohol containing 5 per cent of sulfuric acid. The high boiling fraction was re-esterified. The yield was 95 per cent of the theory. The boiling point given by Paal and Hoffmann[12] is 177°C. (uncorrected). Their analytical data are not entirely satisfactory. The boiling point found on distilling several large lots was 182.7°C. (corrected) at 750 mm. pressure.

0.0978 gm. substance gave 0.2440 gm. CO_2 and 0.0998 gm. H_2O.

	Calculated for $C_9H_{18}O_2$:	Found:
C	68.29	68.05
H	11.48	11.43

5-Methylhexylic Amide.—The acid chloride was prepared by heating 8 gm. of 5-methylhexylic acid with 7.3 gm. of thionyl chloride. The acid chloride was then poured into ice-cold ammonium

hydroxide. The amide was filtered off, dissolved in ether, and the solution filtered. The ether was removed and the amide crystallized. Benzene was found an unsatisfactory solvent as eight crystallizations from this solvent were necessary for purification. Melting point 103.5–104.0°C. (corrected).

0.1013 gm. substance gave 0.2414 gm. CO_2 and 0.1074 gm. H_2O.

	Calculated for $C_7H_{15}NO$:	Found:
C	65.04	64.99
H	11.71	11.87

5-Methylhexyl Alcohol.—The alcohol was prepared by reducing 30 gm. lots of 5-methylhexylic ester with sodium and alcohol in the following manner. The reductions are carried out in a 500 cc. wide-necked, round-bottomed flask fitted with a mechanical stirrer as shown in Fig. 1. The upper half of the mercury seal revolves and is the only part of the apparatus to which the stirring rod is attached. 50 cc. of toluene, dried over sodium, are placed in the flask with six equivalents of metallic sodium. The flask is heated until the toluene refluxes. The stirrer is then rotated vigorously until the melted sodium is divided into a fine emulsion with the toluene. Then while the stirrer is still running, the metal bath is removed and a few cc. of absolute alcohol are allowed to run into the flask through the dropping funnel. Following this the ester, dissolved in three equivalents of alcohol, is rapidly introduced through the same funnel. The best results are obtained if all of this solution is added within 10 or 15 minutes. It is at first necessary to cool the reaction flask with a bath of cold water which is removed as soon as the reaction moderates to such a degree that the reflux condenser is capable of condensing the alcohol vapor. When refluxing has nearly ceased—after 20 to 30 minutes—more absolute alcohol is slowly added. When this no longer causes refluxing the flask is again heated with a metal bath. This is continued with the occasional addition of alcohol until the sodium is completely dissolved. At this time the contents of the flask are entirely in solution. The reaction should be completed in about an hour. If it proceeds more rapidly than this some ester may escape reduction or saponification and may appear as an impurity of the higher alcohol. It is always essential to have a considerable degree of

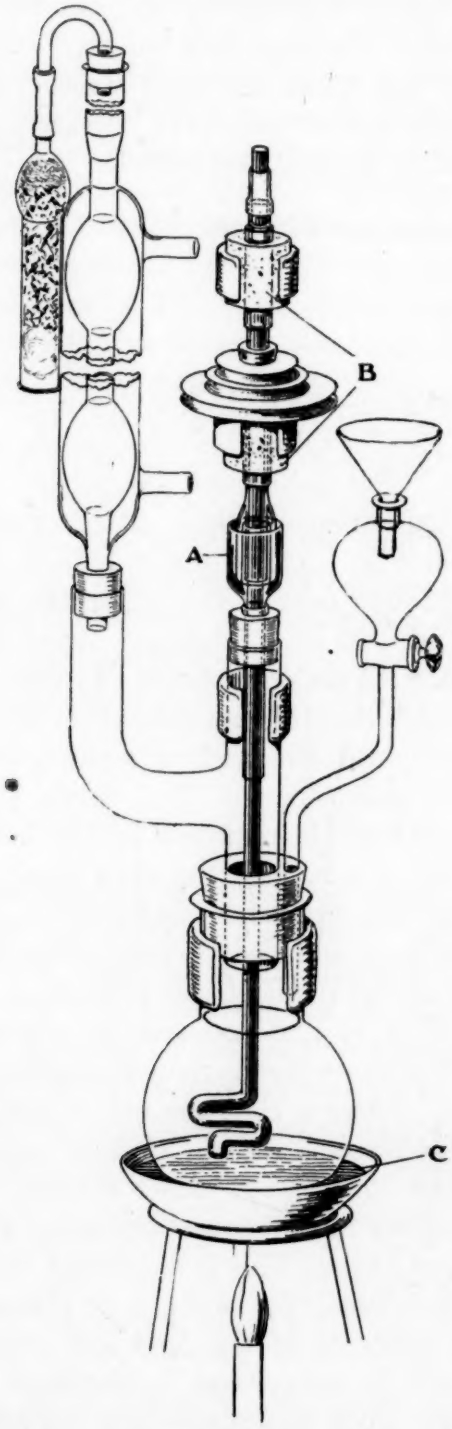


FIG. 1. Apparatus for the reduction of esters to alcohols.

dilution as otherwise the yield of the new alcohol is impaired through side reactions. The quantity of absolute alcohol used for the reduction is generally about 250 cc. The toluene aids the reaction not only by permitting the melting and emulsification of the sodium but also by dissolving the soap formed in course of the reaction, thus keeping the entire mass liquid.

After the reduction is completed the liquid is cooled, diluted with cold water, and rapidly distilled with steam. The first portions of the distillate contain practically pure ethyl alcohol and toluene. When the greater portion of these has been distilled over and the upper layer of liquid in the distilling flask has been reduced to a small volume the further distillate acquires the odor of the new alcohol. The receiver is then changed and soon the distillate turns cloudy and contains water and the new alcohol with little ethyl alcohol and with little or no toluene. Occasionally after all of the new alcohol has been distilled over, a small amount of dark colored material remains floating in the distilling flask. This consists of condensation products and other reduction products from the ester and does not appear if the reduction has been properly carried out. It is not necessary to remove it before recovering the acid resulting from saponification of the ester. This is done by acidifying and subsequently distilling the acid out with steam.

The alcohol-toluene distillate was fractionated through a five bulb 18 inch column. All but a small portion distilled at 78–79°. The residue left in the distilling flask after the temperature has reached 80° is added to the distillate containing the new alcohol. From 5 to 10 per cent of the yield is obtained in this way.

With alcohols of six to nine carbons the yield is 60 to 65 per cent of the theory, calculated on the basis of the ester used. If the recovered acid is taken into consideration the yield is 90 to 95 per cent. Lauric ester gave a slightly smaller yield.

5-Methylhexyl alcohol is very slightly soluble in water and in constant boiling hydriodic acid. Boiling point 170.5°C. (corrected) at 755 mm. pressure. Specific gravity at 25°, 0.8192. A 1.6 cc. pyknometer was used.

0.1018 gm. substance gave 0.2710 gm. CO_2 and 0.1248 gm. H_2O.

	Calculated for $C_7H_{15}O$:	Found:
C	72.33	72.53
H	13.88	13.74

5-Methylhexyl Phenylurethane.—The urethane was prepared from 3 gm. of alcohol in the manner described under 3-methylbutyl phenylurethane. The melting point was constant after three crystallizations from dilute alcohol. Melting point 82.5°C. (corrected).

0.1993 gm. substance neutralized 8.41 cc. 0.1 N HCl (Kjeldahl).

	Calculated for $C_{14}H_{21}NO_2$:	Found:
N	5.95	5.91

5-Methylhexyl Iodide.—75 gm. lots of 5-methylhexyl alcohol were converted into the iodide in the manner described under 3-methylbutyl iodide. The yield was 85 per cent of the theory. The iodide is to a large degree soluble in absolute methyl alcohol. Boiling point 195.0–195.2°C. (corrected) at 765 mm. pressure.

0.1921 gm. substance gave 0.1998 gm. AgI (Carius).

	Calculated for $C_7H_{15}I$:	Found:
I	56.15	56.20

Ethyl 5-Methylhexylmalonate.—5-Methylhexyl iodide was condensed with malonic ester in 50 gm. lots. A portion of the ester was washed with water, dried, and distilled three times under diminished pressure. Boiling point 155°C. (corrected) at 15 mm. pressure.

0.1237 gm. substance gave 0.2964 gm. CO_2 and 0.1134 gm. H_2O.

	Calculated for $C_{11}H_{20}O_4$:	Found:
C	65.06	65.25
H	10.15	10.26

5-Methylhexylmalonic Acid.—The crude ester was saponified and the acid recovered from the soap in the manner described. The lesser solubility in water of 5-methylhexylmalonic acid and of its calcium salt made the use of the continuous extractors unnecessary. The acid is somewhat more soluble in petroleum ether than is 3-methylbutylmalonic acid, but this solvent was still found satisfactory for washing the crystals. The substance crystallizes readily from benzene. Melting point 100.0–100.3°C. (corrected) without decomposition.

0.1380 gm. substance gave 0.2997 gm. CO_2 and 0.1131 gm. H_2O.
0.6218 " " neutralized 61.58 cc. 0.1 N NaOH.

	Calculated for $C_{10}H_{18}O_4$:	Found:
C	59.35	59.20
H	8.96	9.17
Mol. Wt.	202.1	201.9

0.5 gm. of the acid was shaken with 50 cc. of water for 4 hours on 2 consecutive days and then allowed to settle over night. A portion of the clear solution was removed, passed rapidly through a filter and titrated. 10 cc. of solution neutralized 4.11 cc. 0.1 N NaOH, which is equivalent to 0.0415 gm. of the acid. The solubility at 24.5° is therefore 4.15 gm. per liter.

7-Methyloctylic Acid.—The 5-methylhexylmalonic acid was decomposed in the usual manner. After the first distillation the 7-methyloctylic acid was neutralized with sodium hydroxide and the solution extractedt wice with ether to remove impurities. The acid was then recovered from the salt and distilled twice under diminished pressure. It is very slightly soluble in water and is somewhat volatile with steam. Boiling point 248°C. (corrected) at 765 mm. pressure and 140.5°C. at 15 mm. pressure. The acid solidifies in the refrigerator kept at 0°C.

0.1566 gm. substance gave 0.3925 gm. CO_2 and 0.1622 gm. H_2O.

	Calculated for $C_9H_{18}O_2$:	Found:
C	68.29	68.35
H	11.47	11.59

7-Methyloctylic Amide.—The amide was prepared as described under 5-methylhexylic amide and was purified by crystallizing from alcohol. Melting point 106.5° (corrected).

0.1024 gm. substance neutralized 6.50 cc. 0.1 N HCl.

	Calculated for $C_9H_{19}NO$:	Found:
N	8.90	8.88

Ethyl 7-Methyloctylate.—The acid was esterified with alcohol and sulfuric acid in the usual manner. Another sample was esterified by saturating an alcoholic solution of the acid with hydrogen chloride. The yield of distilled ester in each case was about 90 per cent of the theory. 214 gm. of acid gave 228 gm. of ester. Boiling point 220.5°C. (corrected) at 764 mm. pressure.

0.1237 gm. substance gave 0.3208 gm. CO_2 and 0.1326 gm. H_2O.

	Calculated for $C_{11}H_{22}O_2$:	Found:
C	70.90	70.72
H	11.91	12.00

7-Methyloctyl Alcohol.—7-Methyloctylic ester was reduced in the manner described above. 125 gm. of ester gave 62 gm. of alcohol. 30 gm. of crude 7-methyloctylic acid were recovered. The yield of alcohol was 64 per cent. The yield taking the recovered acid into consideration is 89 per cent. Boiling point 206.0°C. (corrected) at 761 mm. pressure. The specific gravity at 25°C. is 0.8260 (1.6 cc. pyknometer was used).

0.1201 gm. substance gave 0.3292 gm. CO_2 and 0.1493 gm. H_2O.

	Calculated for $C_9H_{20}O$:	Found:
C	74.92	74.74
H	13.98	13.92

7-Methyloctyl Phenylurethane.—The urethane was prepared from 2 gm. of 7-methyloctyl alcohol and 1.8 gm. of phenylisocyanate. After three recrystallizations the melting point showed no further change. Melting point 66.4°C. (corrected).

0.2006 gm. substance neutralized 7.45 cc. 0.1 N HCl (Kjeldahl).

	Calculated for $C_{16}H_{25}NO_2$:	Found:
N	5.32	5.20

7-Methyloctyl Iodide.—The first lot of iodide was prepared by very slowly distilling the alcohol with five equivalents of constant boiling hydriodic acid. As the iodide did not distill over readily, in later preparations the acid and alcohol were refluxed for 4 hours and then the iodide was removed in a separatory funnel. The yield was 94 per cent of the theory. 35 gm. of alcohol gave 58 gm. of distilled iodide. Boiling point 120.0°C. (corrected) at 20 mm. pressure.

0.3055 gm. substance gave 0.2806 gm. AgI (Carius).

	Calculated for $C_9H_{19}I$:	Found:
I	49.90	49.65

Ethyl 7-Methyloctylmalonate.—71 gm. of 7-methyloctyl iodide were condensed with malonic ester in two lots. One and a half

equivalents each of sodium and malonic ester were used to avoid as much as possible the formation of di-7-methyloctylmalonic ester. The yield was 65 per cent of the theory. Boiling point 182°C. (corrected) at 18 mm. pressure.[13]

0.1006 gm. substance gave 0.2480 gm. CO_2 and 0.0970 gm. H_2O.

	Calculated for $C_{16}H_{30}O_4$:	Found:
C	67.08	67.23
H	10.56	10.79

7-Methyloctylmalonic Acid.—The ester was saponified and the dibasic acid recovered from the soap in the usual manner. The very small yield of calcium salt from the mother liquors indicated that 7-methyloctylmalonic acid is practically insoluble in water. It crystallizes readily from benzene and is more soluble in petroleum ether than 5-methylhexylmalonic acid. Melting point 92°C. (corrected) without decomposition.

0.1055 gm. substance gave 0.2426 gm. CO_2 and 0.0930 gm. H_2O.

	Calculated for $C_{12}H_{22}O_4$:	Found:
C	62.56	62.70
H	9.63	9.86

9-Methyldecylic Acid.—57 gm. of 7-methyloctylmalonic acid were decomposed and the 9-methyldecylic acid was separated from alkali-insoluble impurities in the usual manner. The monobasic acid was then distilled three times under diminished pressure. It crystallizes in waxy plates at room temperature. It is slightly, if at all, volatile with steam. Boiling point 174.0–174.5°C. (corrected) at 23 mm. pressure.

0.1016 gm. substance gave 0.2650 gm. CO_2 and 0.1088 gm. H_2O.

	Calculated for $C_{11}H_{22}O_2$:	Found:
C	70.90	71.13
H	11.91	11.98

2-Methylpropyl Alcohol (*Isobutyl Alcohol*).—Two 1 kilo lots of Kahlbaum's isobutyl alcohol were used. They had no optical activity and when slowly distilled through a five bulb 18 inch column had a boiling point range of 106–108°. This alcohol was fractionated three times and the fraction boiling at 106–107° used.

[12] It is recognized that to follow the homologous series closely this boiling point should be 2° higher or the boiling point of 6-methylhepthylmalonic ester should be lower. These two boiling points were, however, carefully checked several times. Over 40 gm. of substance were in each case available.

5 pounds of isobutyl alcohol prepared by the Du Pont Chemical Works were also used. This alcohol was purer than the Kahlbaum product. It had no optical activity and 85 per cent of it distilled at 106–107° on the first fractionation. This portion again fractionated through a five bulb column distilled completely at 106–107° (uncorrected).

4-Methylpentylic Acid.—Isobutyl iodide was prepared in the usual manner. All of the iodide was carefully fractionated through a five bulb column. That prepared from the Kahlbaum alcohol was found to contain some lower iodides but was obtained pure after three fractionations. The iodide prepared from the Du Pont alcohol was found to be very pure and was only fractionated once. The iodide used all boiled at 118–119° (uncorrected).

The iodide was condensed with malonic ester in 100 gm. lots. The isobutylmalonic ester was saponified in lots as large as 1 kilo in the manner described above. The soap was thoroughly extracted with acetone twice and then decomposed and the isobutylmalonic acid recovered in the usual manner. The large yield of calcium salt indicated that isobutyl malonic acid is readily soluble in water. As the calcium salt makes a 5 per cent solution in water the process is somewhat wasteful. The yield of crystalline isobutylmalonic acid was 50 per cent of the theory, calculated on the basis of the isobutyl iodide used. The dibasic acid was decomposed and the 4-methylpentylic acid freed from alkali-insoluble impurities in the usual manner. Boiling point, 199.2–199.7° (corrected). A sample of 4-methylpentylic acid prepared from 3-methylbutyl alcohol through the nitrile showed the same boiling point.

4-Methylpentyl Alcohol.—This alcohol has been prepared by several workers[14] and several different boiling points have been given for it.

4-Methylpentylic acid was esterified and the ester reduced in the usual manner. No record was kept of the yield of alcohol which was, however, known to be as good as in the other reductions. Through an oversight the alcohol was not analyzed. As the quantity prepared was large the boiling point given is probably correct. Boiling point 153°C. (corrected) at 760 mm. pressure.

[14] Rossi, A., *Ann. Chem.*, 1865, cxxxiii, 180. Bouveault and Blanc, *Bull. Soc. chim.*, 1904, xxxi, 1210; *Chem. Zentr.*, 1905, 25. Buelens, A., *Bull. Acad. roy. Belg.*, 1908, 921; *Chem. Zentr.*, 1909, 832.

4-Methylpentyl Phenylurethane.—The urethane was prepared from 3 gm. of the alcohol in the usual manner. After six crystallizations from dilute alcohol its melting point showed no further change. Melting point 48°C. (corrected).

0.2010 gm. substance neutralized 9.07 cc. 0.1 N HCl (Kjeldahl).

	Calculated for $C_{13}H_{19}NO_2$:	Found:
N	6.33	6.32

4-Methylpentyl Iodide.—The iodide was prepared and purified in the usual manner. The yield was 68 per cent of the theory, not allowing for the iodide obtained by redistilling the low boiling portions of iodide with acid. Boiling point 173.2°C. (corrected) at 762 mm. pressure.

0.2087 gm. substance gave 0.2326 gm. AgI (Carius).

	Calculated for $C_6H_{13}I$:	Found:
I	59.95	60.25

Ethyl 4-Methylpentylmalonate.—The iodide was condensed with malonic ester in 50 gm. lots. A portion of the ester was purified and gave the following values. Boiling point 137.0°C. (corrected) at 11 mm. pressure.

0.0994 gm. substance gave 0.2320 gm. CO_2 and 0.0993 gm. H_2O.

	Calculated for $C_{13}H_{24}O_4$:	Found:
C	63.88	63.65
H	9.91	10.20

4-Methylpentylmalonic Acid.—The crude ester was saponified, the soap washed with acetone, and the acid recovered in the usual manner. The acid is moderately soluble in water, crystallizes well from benzene, and is quite insoluble in petroleum ether. A portion after three crystallizations from benzene showed no further change in melting point. Melting point 86.5°C. (corrected) without decomposition.

0.1280 gm. substance gave 0.2696 gm. CO_2 and 0.0993 gm. H_2O.

	Calculated for $C_9H_{16}O_4$:	Found:
C	57.41	57.44
H	8.57	8.71

6-Methylheptylic Acid.—The dibasic acid was decomposed and the monobasic acid freed from alkali-insoluble impurities in the usual manner. The 6-methylheptylic acid was then distilled twice under diminished pressure. Boiling point 126–127°C. (corrected) at 14 mm. pressure and 232°C. at 762 mm. pressure. It solidifies in a refrigerator kept at 0°C. A sample of the acid prepared from 5-methylhexyl iodide through the nitrile gave the same boiling point.

0.1500 gm. substance gave 0.3648 gm. CO_2 and 0.1478 gm. H_2O.

	Calculated for $C_8H_{16}O_2$:	Found:
C	66.58	66.32
H	11.18	11.04

6-Methylheptylic Amide.—8 gm. of 6-methylheptylic acid were converted into the acid chloride and then into the amide in the usual manner. The substance crystallized in white glistening plates. The constant melting point is 114.0°C. (corrected).

0.1002 gm. substance gave 0.2459 gm. CO_2 and 0.1068 gm. H_2O.

	Calculated for $C_8H_{17}ON$:	Found:
C	67.06	66.92
H	11.97	11.94

Ethyl 6-Methylheptylate.—The acid was esterified with 99.5 per cent alcohol and sulfuric acid. The ester was washed with water, dried, and distilled five times. Boiling point 200.3°C. (corrected) at 761 mm. pressure.

0.1394 gm. substance gave 0.3552 gm. CO_2 and 0.1473 gm. H_2O.

	Calculated for $C_{10}H_{20}O_2$:	Found:
C	69.70	69.50
H	11.71	11.83

6-Methylheptyl Alcohol.—6-Methylheptylic ester was reduced in the usual manner. Difficulty in purifying the alcohol brought the yield of distilled alcohol down to 80 per cent, taking into consideration the recovered acid. 144 gm. of ester gave 71 gm. of alcohol or 65 per cent of the theory. Boiling point 188.5°C. (corrected) at 764 mm. pressure. Specific gravity at 25°, 0.8230 (1.6 cc. pyknometer used).

0.1180 gm. substance gave 0.3179 gm. CO_2 and 0.1476 gm. H_2O.

	Calculated for $C_8H_{17}O$:	Found:
C	73.77	73.47
H	13.94	14.01

6-Methylheptyl Phenylurethane.—3 gm. of alcohol were converted into the urethane in the usual manner. After three crystallizations from alcohol the melting point showed no further change. Melting point 81.0–81.4°C. (corrected).

0.1006 gm. substance neutralized 4.05 cc. 0.1 N HCl (Kjeldahl).

	Calculated for $C_{15}H_{23}O_2N$:	Found:
N	5.62	5.64

6-Methylheptyl Iodide.—50 gm. of 6-methylheptyl alcohol were refluxed for 4 hours with five equivalents of constant boiling hydriodic acid. The yield of distilled iodide was 81 gm. or 88 per cent of the theory. It is insoluble in hydriodic acid and makes about a 20 per cent solution in absolute methyl alcohol. Boiling point 100.0°C. (corrected) at 17 mm. pressure.

0.1713 gm. substance gave 0.1671 gm. AgI (Carius).

	Calculated for $C_8H_{17}I$:	Found:
I	52.87	52.73

Ethyl 6-Methylheptylmalonate.—51.5 gm. of 6-methylheptyl iodide were condensed with malonic ester using one and a half equivalents each of sodium and malonic ester. The yield of distilled ester was 42.5 gm. or 73 per cent of the theory. Two distillations under diminished pressure gave a pure sample. Boiling point 171.8°C. at 18 mm. pressure. This boiling point was determined with unusual care.[13]

0.1039 gm. substance gave 0.2523 gm. CO_2 and 0.0950 gm. H_2O.

	Calculated for $C_{15}H_{28}O_4$:	Found:
C	66.12	66.22
H	10.36	10.24

6-Methylheptylmalonic Acid.—The distilled 6-methylheptylmalonic ester was saponified and the soap washed with acetone. The acid was recovered from the soap and recrystallized in the usual manner. It crystallizes well from benzene and is moderately sol-

uble in petroleum ether. Melting point 89.5–90.0°C. (corrected) without decomposition.

0.1020 gm. substance gave 0.2279 gm. CO_2 and 0.0859 gm. H_2O.

	Calculated for $C_{11}H_{20}O_4$:	Found:
C	61.06	60.93
H	9.33	9.43

8-Methylnonylic Acid.—The 6-methylheptylmalonic acid was decomposed and the monobasic acid freed from alkali-insoluble impurities in the usual manner. The 8-methylnonylic acid was distilled three times under diminished pressure. After the first distillation the whole quantity was of practically constant boiling point. The acid solidifies at room temperature. Boiling point 155.6°C. (corrected) at 16 mm. pressure.

0.1242 gm. substance gave 0.3174 gm. CO_2 and 0.1296 gm. H_2O.

	Calculated for $C_{10}H_{20}O_2$:	Found:
C	69.70	69.68
H	11.71	11.69

Oxidation with Hydrogen Peroxide.

The acids employed for the oxidation experiments were in every case ester-free and of constant boiling point. The hydrogen peroxide varied in strength from 2.67 to 3.87 per cent as shown by titration. All the samples of hydrogen peroxide reacted acid to Congo red and contained traces of inorganic matter.

In alkaline solution the oxidations were not successful and little or no acetone was formed. A maximum yield of acetone was obtained when the oxidation was done in slightly acid solution. In those oxidations in which it was desired to obtain aldehydes the distillation was quite rapid. When a maximum yield of acetone was desired the rate of distillation was slower.

Small condensers were used to avoid mechanical loss of aldehydes and acids. The condensers extended below the surface of the liquid in the receivers which were very efficiently cooled with an ice mixture. By this means the loss of material was effectively prevented.

Two series of oxidations were carried out. The first series had for its purpose the preparation and identification of the

aldehydes, volatile acids, and ketones. The second series aimed to show the effect on the yield of acetone of the increasing distance between the tertiary carbon atom and the carboxyl group. In this series the number of distillations was reduced to a minimum.

Preparation of Samples for Oxidation.—A given weight of the acid was mixed with about 10 cc. of water, approximately neutralized with 10 per cent sodium hydroxide, and the calculated amount of hydrogen peroxide added. The mixture was made neutral to phenolphthalein and then about 0.3 cc. of 10 per cent sulfuric acid added.

Procedure for the First Series of Oxidations.—The mixture of soap and hydrogen peroxide was distilled until the volume of the liquid in the flask was reduced to 20 to 30 cc. This residue contained the sodium salts of the unoxidized acid and of any acidic oxidation products, together with inorganic salts. It will be referred to as the oxidation residue.[15] If it showed the presence of formic acid (silver test) a few cc. of 0.5 N potassium permanganate solution were added and the mixture was placed in the refrigerator at 0°C. for a few hours. Following this it was acidified and the acids were extracted with ether in the customary way. Finally the acids were converted into silver salts.

Following the procedure of Dakin, the distillate from the oxidation was separated into two fractions, one containing ketones and aldehydes, and the other the volatile acids. In order to bring about this separation the distillate was rendered alkaline and redistilled. The residue contains the soaps of the volatile acids and the distillate contains the aldehydes and ketones. The volatile acid residue was treated in the same manner as the oxidation residue. In order to separate the aldehydes from the ketones they were oxidized by means of Tollens' reagent into the corresponding acids. After this the mixture was made acid with sulfuric acid and distilled nearly to dryness. This distillate again was rendered slightly alkaline and redistilled until the volume of the liquid in the distilling flask was reduced to 20 to 30 cc. The volatile acids of this final residue were identified in the form of silver salts. They will be referred to as acids

[15] Several unsuccessful attempts were made to isolate dibasic acids from the oxidation residues from 4-methylpentylic and 5-methylhexylic acids. No indication of the presence of these acids was obtained.

from aldehydes. The distillate containing the ketones was extracted twice with ether. For identification of the acetone the extracted solution was treated with a solution of *p*-nitrophenylhydrazine. The reagent was prepared, according to the method of Dakin,[16] by dissolving one part of the hydrazine in thirty parts of hot 40 per cent acetic acid and then cooling and filtering the solution. The hydrazone which precipitated almost at once was filtered off and crystallized from alcohol until pure.

Procedure for the Second Series of Oxidations.—3 gm. lots of acid were prepared for oxidation in slightly acid solution as described above. Three and a half equivalents of hydrogen peroxide were used. The mixture was distilled until the volume of liquid in the distilling flask was reduced to 20 to 30 cc. The aldehydes in the distillate were oxidized with Tollens' reagent. The mixture was then made acid with strong sulfuric acid and distilled almost to dryness. The new distillate which contains volatile acids and ketones was used for the estimation of acetone. It was separated into two parts and one part was employed for titration directly. The other part was extracted with one-fourth its volume of alcohol-free ether prior to titration. The object of this treatment was to remove the difficulty which was encountered in the titration of the ketone fraction obtained in the experiments with acids higher than isocaproic. The ketones formed as the result of β oxidation in these experiments, owing to their insolubility in water, make it difficult to obtain a uniform sample for titration. It was established by preliminary experiment that an aqueous solution of acetone of a concentration approaching that of our experiments loses under these fixed conditions between 11 and 14 per cent of the original acetone. It is recognized that in the case of the higher acids the accuracy of the data obtained for the unextracted solutions is open to question. Nevertheless the values reported are the result of titrations which check closely and the results are serviceable for comparison.

The acetone was titrated by the Messinger-Huppert iodine-thiosulfate method. It was found necessary to carry out the titration in a large and uniform volume of liquid to avoid the

[16] Dakin, *J. Biol. Chem.*, 1908, iv, 235.

errors otherwise arising from the heat of neutralization of the 10 cc. of 40 per cent sodium hydroxide used in the process. 10 cc. lots of the distillates containing from 5 to 20 mg. of acetone were diluted to 400 cc. and the titration was carried out in the usual manner. 1 cc. of 0.1 N iodine is equivalent to 0.967 mg. of acetone.

Oxidation of Isobutyric Acid (Second Series).—Kahlbaum's isobutyric acid was distilled twice and the fraction boiling at 155.2–155.5°C. (corrected) was used. The acid was oxidized in the manner described above and the following data were obtained.

TABLE II.

Total volume.	Volume titrated.	0.1 N I used.		Acetone.	
		Before extraction.	After extraction.	Before extraction.	After extraction.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
250	10	13.25	11.75	320	284
250	10	12.45	11.05	310	267
263	10	9.45	8.55	240	218

Another series of isobutyric acid oxidations was carried out to prove the formation of volatile acids of lower molecular weight.

5 gm. lots of isobutyric acid were prepared for oxidation in the usual manner. Only one equivalent of hydrogen peroxide was used. The distillates from the oxidations were made alkaline and redistilled. The residue from this distillation contains isobutyric acid and the lower acids formed by the oxidation. Silver salts were made from the residues and gave the following values. The absence of formic acid was indicated by these salts being entirely stable. The molecular weights were determined on the basis of the silver content.

Sample.	Silver.	Mol. Wt.	
		Found:	Calculated for $C_4H_7O_2Ag$:
mg.	*mg.*		
0.0559	0.0332	179.4	180.9
0.1233	0.0735	181.0	
0.1433	0.0815	189.9	

Oxidation of 3-Methylbutylic Acid (Second Series).—Pure 3-methylbutylic acid was prepared by the oxidation of 3-methylbutyl alcohol with sodium bichromate. The acid was oxidized in the manner described above. The following data were obtained.

TABLE III.

Total volume.	Volume titrated.	0.1 N I required.		Acetone.	
		Before extraction.	After extraction.	Before extraction.	After extraction.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
200	10	21.2	18.8	410	364
215	10	20.5	17.7	426	368

Oxidation of 4-Methylpentylic Acid (First Series).—5 gm. lots of 4-methylpentylic acid were oxidized as described under the procedure for the first series of oxidations. Table IV gives the analytical data obtained.

TABLE IV.

Source of silver salt.	Sample.	Silver.	Mol. Wt.	
			Found:	Calculated for $C_5H_{11}O_2Ag$:
	mg.	*mg.*		
Oxidation residue.....	0.1794	0.0867	223.1	222.9
" "	0.1966	0.0951	223.1	
" "	0.1633	0.0784	224.7	
Volatile acids.....	0.2013	0.0965	225.2	222.9
" "	0.1861	0.0898	223.6	
				$C_5H_9O_2Ag$:
Acids from aldehydes.	0.1332	0.0697	206.2	208.9
" " "	0.1552	0.0816	205.3	
" " "	0.0131	0.0068	207.9	

The *p*-nitrophenylhydrazone of acetone obtained was crystallized twice from alcohol. Melting point 148.5–149.0°C (corrected).

0.1366 gm. substance gave 0.2798 gm. CO_2 and 0.0729 gm. H_2O.

	Calculated for $C_9H_{11}O_2N_3$:	Found:
C.....	55.95	55.85
H.....	5.74	5.97

Oxidation of 4-Methylpentylic Acid (Second Series).—The acid was oxidized as described under the procedure for the second series of oxidations. The following analytical data were obtained.

TABLE V.

Total volume.	Volume titrated.	0.1 N I required.		Acetone.	
		Before extraction.	After extraction.	Before extraction.	After extraction.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
200	10	15.7	12.95	306	205
250	10	12.95	10.95	313	265
200	10	14.0	11.55	271	223

Oxidation of 5-Methylhexylic Acid (First Series).—Since a considerable amount of 5-methylhexylic acid was available the oxidations were carried out on larger quantities. The results show a normal increase in the yield of oxidation products. The oxidations were carried out as described under the procedure for the first series of oxidations. 5 gm. lots of acid were oxidized with five equivalents of hydrogen peroxide. Table VI gives the analytical data obtained.

TABLE VI.

Source of Ag salt.	Sample.	Silver.	Mol. Wt.	
			Found:	Calculated for $C_7H_{13}O_2Ag$:
	mg.	*mg.*		
Oxidation residue.....	0.1983	0.0904	236.7	236.9
" "	0.1624	0.0742	236.0	
Volatile acids.....	0.1002	0.0466	232.1	236.9
" "	0.1857	0.0847	236.5	
				$C_6H_{11}O_2Ag$:
Acids from aldehydes.	0.1613	0.0783	222.1	222.9
" " " .	0.2110	0.1017	223.8	

The *p*-nitrophenylhydrazone of acetone obtained was crystallized three times from alcohol. Melting point 149.2°C. (corrected).

Oxidation of 5-Methylhexylic Acid (Second Series).—The acid was oxidized as described under the procedure for the second series of oxidations. Table VII gives the analytical data obtained.

TABLE VII.

Total volume.	Volume titrated.	0.1 N I required.		Acetone.	
		Before extraction.	After extraction.	Before extraction.	After extraction.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
200	10	12.45	9.25	241	179
200	10	12.55	9.25	243	179
200	10	17.30	13.05	335*	252*
250	10	14.25	10.65	344*	257*

* These oxidations were carried out under different conditions.

Oxidation of 6-Methylheptylic Acid (Second Series).—The acid was oxidized in the manner described above and the following analytical data were obtained.

TABLE VIII.

Total volume.	Volume titrated.	0.1 N I required.		Acetone.	
		Before extraction.	After extraction.	Before extraction.	After extraction.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
200	10	11.5		222	
220	10	10.3		219	
213	10	12.45	9.45	256*	195*
210	10	12.65	9.3	257*	189*
200	10		8.25		159
200	10		7.70		149
160	10		9.2		142

* These oxidations were carried out under different conditions.

After titration all solutions were combined and extracted twice. The extracted solution was used for the preparation of the *p*-nitrophenylhydrazone of acetone. The hydrazone was crystallized three times from alcohol. Melting point 148.5–149.5°C. (corrected).

0.1003 gm. substance gave 0.2049 gm. CO_2 and 0.0535 gm. H_2O.

	Calculated for $C_9H_{11}N_3O_2$:	Found:
C	55.95	55.71
H	5.74	5.97

Oxidation of 7-Methyloctylic Acid (Second Series).—The acid was oxidized in the manner described above. Table IX gives the analytical data obtained.

TABLE IX.

Total volume.	Volume titrated.	0.1 N I required.		Acetone.	
		Before extraction.	After extraction.	Before extraction.	After extraction.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
200	10	4.85	4.02	93	78
200	10	6.92	5.12	134	99

To secure a larger amount of acetone the oxidation residue left after the first distillation was oxidized twice more with three equivalents of hydrogen peroxide in the usual manner. All the distillates containing acetone were combined and extracted twice with ether. The solution was then distilled until about one-third of it had passed over. The new distillate was used for making the *p*-nitrophenylhydrazone. The residue from the distillation gave no test for acetone. The hydrazone was crystallized three times from alcohol. Melting point 148.5–149.5°C. (corrected).

Oxidation of 9-Methyldecylic Acid (Second Series).—The acid was oxidized in the manner described above. Table X gives the analytical data obtained.

TABLE X.

Total volume.	Volume titrated.	0.1 N I required.		Acetone.	
		Before extraction.	After extraction.	Before extraction.	After extraction.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
200	10	3.72	2.72	72	53
200	10	3.72	2.72	72	53

The oxidation residues were oxidized twice more and the acetone *p*-nitrophenylhydrazone was prepared as described under the oxidation of 7-methyloctylic acid. Only a small quantity of hydrazone was obtained. This was crystallized twice from alcohol. Melting point 146–148°C. (corrected).

TABLE XI.

Average of Data from the Second Series of Oxidations.

Acid.	Acetone.							Loss on extraction.
	Theoretical yield.	Before extraction.			After extraction.			
		Actual yield.	Per cent of theory.	Yield per gm. mol.	Actual yield.	Per cent of theory.	Yield per gm. mol.	
	mg.	*mg.*		*mg.*	*mg.*		*mg.*	*per cent*
2-Methylpropylic .	1,978	290	14.6	851	258	13.0	757	11.0
3-Methylbutylic...	1,707	418	24.5	1,421	366	21.4	1,245	12.4
4-Methylpentylic..	1,500	297	19.8	1,149	246	16.4	952	17.2
5-Methylhexylic ...	1,338	242	18.2	1,049	179	13.4	776	25.4
6-Methylheptylic ..	1,208	220	18.2	1,056	150	12.4	720	25.0*
7-Methyloctylic...	1,101	114	10.3	600	89	8.1	469	22.0
9-Methyldecylic....	936	72	7.7	447	53	5.7	311	26.4

* Calculated from the data marked with an asterisk under oxidation of 6-methylheptylic acid (Table VIII).

THE MECHANISM OF CHOLESTEROL ABSORPTION.

By J. HOWARD MUELLER.

(From the Department of Pathology of the College of Physicians and Surgeons, Columbia University, New York.)

(Received for publication, September 25, 1916.)

By the analysis of chyle obtained from thoracic duct fistulas in dogs cholesterol which was fed has been shown[1] to be absorbed from the alimentary tract through the lymphatic system, whether fed as free cholesterol or as cholesterol esters. An increase in both fractions was always recognizable in the chyle, so that the normal proportion of one part of free to two or three parts of combined was quite constantly maintained. It seemed desirable to investigate further the mechanism of this process, particularly since, as has been pointed out,[2] there are rather important theoretical reasons for studying the process of a physiological interchange between free and combined cholesterol.

There are three general locations in which the esterification or saponification processes can take place during the absorption of cholesterol and its esters. *A priori* it might be expected from the general chemical and physical similarities between these bodies and the neutral fats that the same enzymes and tissues which are connected with the absorption of the latter must also take part in that of the former. The enzymes within the gastro-intestinal tract, the intestinal mucosa, and the mesenteric lymph nodes[3] all are concerned in the absorption of neutral fats. Cholesterol, during its absorption into the chyle of the thoracic duct, must be subjected to the activities of the same enzymes and tissues. The experiments to be described have been directed at all these agencies; and while it may be

[1] Mueller, J. H., *J. Biol. Chem.*, 1915, xxii, 1.

[2] Mueller, *J. Biol. Chem.*, 1916, xxv, 561.

[3] Stheeman, H. A., *Beitr. path. Anat. u. allg. Path.*, 1910, xlviii, 170.

stated here that the results do not furnish sufficient evidence for a complete explanation of the changes which apparently take place, yet it is thought they may throw some little light on the process.

Influence of the Gastro-Intestinal Tract on Cholesterol Absorption.

A possible change in the ingested cholesterol taking place in the stomach or intestine was sought for in three general ways: (1) by analysis of the gastric and intestinal contents after feeding cholesterol; (2) by following the absorption through a thoracic duct fistula with experimental elimination of certain of the digestive juices; and (3) by experiments *in vitro* dealing with the possible action of various enzymes upon cholesterol.

The determination of cholesterol was carried out by the Fraser and Gardner[4] modification of the Windaus[5] digitonin method. The extract of the material was prepared in the case of fluids, such as chyle, by the alcohol-ether method of Bloor,[6] and in the case of tissues, by treating the finely divided material with hot alcohol-ether followed by several extractives with boiling alcohol. To remove water-soluble extractives, which are always present, the combined extracts were evaporated to a small bulk, water and salt added (to prevent formation of emulsions), and the material extracted thoroughly with ether. The details of this method I have discussed elsewhere.[7]

Experiment 1.—After 24 hours' fasting, a dog was allowed to eat four raw eggs, shaken up in 350 cc. of water to which a little NaCl had been added. 10 cc. of this egg mixture were reserved for analysis. After 2 hours the stomach contents were removed by tube. A 20 cc. sample was analyzed. Peptic digestion had apparently been active, for the precipitate formed on adding alcohol to the digested sample was very light and fine compared to the heavy curdy precipitate of the fresh mixture.

[4] Fraser, M. T., and Gardner, J. A., *Proc. Roy. Soc., Series B*, 1910, lxxxii, 559.

[5] Windaus, A., *Z. physiol. Chem.*, 1910, lxv, 110.

[6] Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.

[7] Mueller, *J. Biol. Chem.*, 1916, xxv, 549.

	gm		
Sample 1.			
Free	0.0236		
Combined	0.0020	C : T*	7.8
Sample 2.			
Free	0.0251		
Combined	0.0027	C : T	9.7

* C : T denotes the ratio of combined cholesterol to total cholesterol on a percentage basis.

Experiment 2.—A dog was allowed to eat a small piece of meat and three raw eggs. 4 hours later it was killed, and the contents of the small and large intestines were removed separately. The small intestine contained about 150 cc. of thick yellow fluid, and the large intestine about 30 cc. of material of the same appearance, together with some dark colored solid feces. The latter were discarded. About 10 cc. of each of the contents of the large and small intestines were analyzed.

	gm.		
Small intestine.			
Free	0.0369		
Combined	0.0016	C : T	4.2
Large intestine.			
Free	0.0227		
Combined	0.0022	C : T	8.8

As far as can be seen from these results, no esterification of free cholesterol appears to take place in the stomach or intestine. It is quite possible, however, in spite of such negative findings, that the ester might be formed slowly and be absorbed practically as fast as formed.

Experiment 3.—A dog was fed bread and milk containing 10 cc. of sesame oil in which 1.25 gm. of cholesterol oleate had been dissolved. After killing the animal 3 hours later, at which time the mesenteric lymphatics were white and prominent, the gastric and intestinal contents were analyzed. The stomach contained a considerable amount of undigested bread mixed with drops of oil. An unweighed portion of this was extracted and analyzed. Only about 5 cc. of a thick, bile-stained fluid could be obtained from the small intestine; this was extracted.

	gm.		
Stomach.			
Free	0.0139		
Combined	0.0256	F : T*	35
Intestine.			
Free	0.0360		
Combined	0.0077	F : T	82

* F: T denotes the ratio of free cholesterol to total cholesterol on a percentage basis.

These figures seem to indicate that hydrolysis of the ester takes place in the intestine. In view of experiments to be described later, however, this interpretation is not so probable, and it is well to note that if there were a selective absorption of the esters from the intestine, the result would be the same. Possibly only a part of the meal had reached the intestine, for while the whole of the intestinal contents was analyzed, the total cholesterol amounting only to some 0.04 gm., only a part, probably less than one-tenth, of the stomach contents was used, so that there was present in the stomach perhaps 0.5 gm. of cholesterol. Since a considerable part of the cholesterol present in the intestine must have come from the bile, evidently there was too little ingested cholesterol present to warrant any definite conclusions from this experiment.

By studying the absorption of cholesterol through the thoracic duct after experimentally diverting a single secretion, such as bile or pancreatic juice, from the intestine, it was hoped to gain an insight into the part played by such enzymes in the process.

Experiment 4.—Immediately after feeding a dog a small piece of meat, the animal was etherized, a thoracic duct fistula produced, and a sample of chyle collected. Then, through a laparotomy incision, 10 cc. of a solution of egg white, containing a small amount of dilute hydrochloric acid, were injected by means of a large hypodermic needle, directly into the duodenum. It was hoped in this way to start the normal digestive secretions into the intestine. This injection was followed by 10 cc. of cottonseed oil, containing 2 gm. of cholesterol oleate, given in the same way. The dog recovered promptly from the ether. After 2 hours chyle was obtained which was only moderately cloudy, and 200 cc. of milk were given the dog. In 2 hours more chyle which was very milky was collected and analyzed.

		Free.	Combined.	F : T.
		per cent	*per cent*	
11.00 a.m.	Thoracic duct fistula completed. Chyle collected.....	0.022	0.038	36
11.30 "	Oil injected into intestine.			
1.30 p.m.	Chyle collected, moderately milky. Fed 200 cc. of milk.			
3.30 "	Chyle collected, very milky.			
5.30 "	" " " "	0.065	0.185	26

That gastric digestion is not an essential feature in the absorption of cholesterol is shown by the results of this experiment. Perfectly normal, if somewhat delayed, absorption took place when the solution of cholesterol in oil was injected directly into the duodenum. The delay probably resulted from a failure of the hydrochloric acid injection to bring about the pancreatic secretion, so that only after starting the normal sequence by feeding a little milk was the intestinal digestion properly started.

In the experiments now to be described, in which the bile was excluded from the intestine, the same general plan was followed in each case. The common bile duct of the dog was ligated, under ether, the gall bladder was brought up into the wound, a rubber tube, with a projection on its inner end formed by rolling it back upon itself, was fastened into the gall bladder by a purse string suture, and the wound was closed. Bile was removed three or four times a day through this cannula; at other times it was closed by means of a small clamp and protected by a close fitting oilcloth coat. In two instances the dogs became somewhat jaundiced on the following day, once due to hemorrhage into the gall bladder, with subsequent clotting and occlusion of the cystic duct; the cause for the other was not determined. Thoracic duct fistulas were made on the 2nd day after the biliary fistulas in order to give time for the bile present in the intestine to be eliminated as far as possible. Feces passed by the dogs during the course of the experiment were clay-colored, indicating the absence of bile.

Experiment 5.

		Free.	Combined.	F : T.
		per cent	*per cent*	
Dec. 30.	Biliary fistula made.			
Jan. 1.				
11.30 a.m.	Thoracic duct fistula completed. Chyle taken.....	0.021	0.028	43
1.30 p.m.	Chyle taken. Fed small piece of meat.			
1.45 "	Fed four raw eggs.			
5.00 "	Chyle taken, very milky.....	0.056	0.038	60
6.00 "	" " " "	0.058	0.037	61

Experiment 6.

Jan. 17.	Biliary fistula made.			
" 19.				
11.15 a.m.	Thoracic duct fistula completed. Chyle taken.....	0.020	0.037	35
5.15 p.m.	Fed three raw eggs.			
8.15 "	Chyle taken, very milky.....	0.054	0.050	52
9.15 "	" " " "	0.050	0.037	57

Experiment 7.

Jan. 29.	Biliary fistula made.			
" 30.	Dog jaundiced (gall bladder contained clotted blood).			
Feb. 1.				
11.00 a.m.	Thoracic duct fistula completed. Chyle taken.....	0.037	0.044	46
12.00 "	Fed small piece of meat and three raw eggs.			
4.00 p.m.	Chyle taken, milky.....	0.051	0.063	45

Experiment 8.

Mar. 4.	Biliary fistula made.			
" 6.				
12.00 m.	Thoracic duct fistula completed.			
2.15 p.m.	Chyle taken. Fed four eggs.	0.022	0.045	33
5.15 "	Chyle collected, milky.....	0.034	0.065	34
6.15 "	" " "	0.035	0.042	45

Experiment 9.

		Free.	Combined.	F : T.
		per cent	*per cent*	
June 6.	Biliary fistula made.			
" 8.				
11.30 a.m.	Thoracic fistula completed. Chyle taken.....	0.047	0.058	44
2.00 p.m.	Chyle taken. Given 200 cc. of milk and three eggs.....	0.045	0.058	44
5.30 "	Chyle taken, very milky.	0.054	0.060	47
8.15 "	" " " "	0.050	0.055	48

Experiment 10.

June 10.	Biliary fistula made.			
" 12.				
10.00 a.m.	Thoracic duct fistula completed.			
2.30 p.m.	Given 200 cc. of milk. Chyle collected.....	0.057	0.094	38
	Fed 1.5 gm. of cholesterol oleate in 10 cc. of olive oil and 200 cc. of water.			
8.45 "	Chyle collected, quite milky..	0.073	0.087	46
	Fed 100 cc. of milk and then 1.5 gm. of cholesterol oleate in 10 cc. of cottonseed oil and 200 cc. of water, plus 3 gm. of dried ox bile.			
11.30 "	Chyle collected, quite milky.....	0.063	0.093	41

Experiment 11.

June 12.	Biliary fistula made.			
" 14.				
10.00 a.m.	Thoracic duct fistula completed.			
1.00 p.m.	Chyle taken. Fed 1 gm. of cholesterol in 10 cc. of cottonseed oil and 200 cc. of milk....	0.038	0.066	36
5.00 "	Chyle taken. Fed 150 cc. of milk and then 1 gm. of cholesterol in 10 cc. of cottonseed oil and 200 cc. of water, plus 1 gm. of sodium taurocholate (Merck)..	0.041	0.065	39
9.00 "	Chyle taken, quite milky	0.057	0.063	48
June 15.				
10.00 a.m.	" " " "	0.062	0.083	43

The bile apparently has a rather important function in connection with cholesterol absorption. Rothschild states[8] that after tying the common bile duct in dogs no cholesterol is absorbed. This evidently does not hold strictly. It each case there is a rise in the total cholesterol after feeding. This rise is small in some cases, and quite pronounced in others, but in no case is it so pronounced as that obtained in normal dogs. It does not depend upon the amount of fatty material absorbed, for in all cases, after feeding, chyle was obtained which was quite milky, the alcohol-ether extracts of which left a considerable amount of oily material after evaporation.

It has been suggested that bile aids the absorption of neutral fats by the reaction of the bile acids with the fatty acids, and in this way makes possible the entrance of the water-insoluble fatty substance into the cells of the mucosa. Cholesterol is also held in solution by bile acids, and its absorption may be facilitated in a similar manner.

The other noticeable fact brought out by the experiments is the relative increase in free cholesterol absorbed. In each case the percentage of free to total cholesterol was above that found normally after feeding. This figure seems to vary normally between perhaps 25 and 35 per cent, while in the biliary fistula animals it was between 39 and 61 per cent. The significance of this variation is not evident.

Normal absorption was not produced by the addition of bile or sodium taurocholate to the diet.

The experiments in which the pancreatic juice was excluded from the intestine were planned in the same way, allowing at least 2 days after the first operation for the dog to recover as far as possible. Under ether, the two pancreatic ducts were ligated and cut, and the pancreas itself, while left *in situ*, was separated from the intestine throughout its entire course, except for the larger blood vessels. In this way, it was believed, only the intestinal secretion would be eliminated, while the far reaching disturbances in carbohydrate metabolism, incident upon a complete removal of the pancreas, would be obviated. All the dogs recovered rapidly from the first operation, and were apparently in good condition when the thoracic duct fistulas were done.

[8] Rothschild, M. A., *Proc. N. Y. Path. Soc.*, 1914, xiv, 229.

Experiment 12.

		Free.	Combined.	F : T.
		per cent	*per cent*	
May 23.	Pancreatic ducts ligated and cut.			
" 27.				
11.00 a.m.	Thoracic duct fistula completed. Chyle taken.....	0.050	0.085	37
2.00 p.m.	Chyle taken. Fed 200 cc. of milk and two eggs.			
4.30 "	Chyle taken.....	0.051	0.074	41
6.00 "	" "	0.058	0.077	43
8.00 "	" "	0.058	0.080	42

Experiment 13.

May 24.	Pancreatic ducts ligated and cut.			
" 27.				
12.00 m.	Thoracic duct fistula completed. Chyle taken.....	0.047	0.084	36
2.00 p.m.	Chyle taken. Fed 2 gm. of cholesterol oleate in 10 cc. of olive oil and the white of an egg in 150 cc. of water.			
4.30 "	Chyle taken, milky.....	0.047	0.075	39
6.00 "	" " "	0.053	0.077	41
8.30 "	" " "	0.057	0.074	43

Experiment 14.

May 29.	Pancreatic ducts ligated and cut.			
June 1.				
12.00 m.	Thoracic duct fistula completed. Chyle taken.			
2.45 p.m.	Chyle taken. Fed 150 cc. of milk and then 2 gm. of cholesterol oleate in 10 cc. of olive oil, in 150 cc. of water containing the white of an egg.	0.050	0.104	33
5.00 "	Chyle taken.....	0.045	0.082	36
8.15 "	" " quite milky.....	0.057	0.083	41

Absence of the pancreatic juice seems to diminish the absorption of cholesterol very greatly. There is a slight increase in the free cholesterol after eating, but the cholesterol esters seem to decrease to some extent. The total cholesterol present remains nearly constant, but the ratio of free to total cholesterol rises somewhat. Here again, as in the case of the bile experiments, the decreased absorption cannot be due entirely to the absence of other lipoids in the chyle, for some of the samples at least were quite milky, although none contained as much fat as is present in normal digestion.

It seems evident that the pancreatic juice, and to a less extent the bile, exert an important influence on the absorption of cholesterol. If there could be demonstrated either a well marked hydrolytic action upon cholesterol esters, or an esterification of free cholesterol by one of the intestinal enzymes, it might help clear the situation. Experiments with this probability in view were undertaken with pancreatic extract, as well as with the intestinal mucosa of the dog. In the case of the pancreas, the organ was put through a meat chopper, and two or three parts of 50 per cent glycerol were added, to prevent as far as possible the activation of trypsin with the possible destruction of a lipase or "cholesterase." This suspension of hashed gland in dilute glycerol was used without filtering. The intestinal mucosa, after being scraped from the small intestine, was suspended in four parts of water. Since both cholesterol and cholesterol oleate are insoluble in aqueous media and solid at incubator temperature, a solution in sesame oil was used. This not only enabled the cholesterol to be presented to the enzyme action in a finely divided form, but also furnished an indication of ordinary lipase activity from the amount of fatty acid set free, which was estimated by titration.

For most of the experiments, four equal portions of 3 to 5 gm. of the ground pancreas or intestinal mucosa were taken, glycerol or water added to each, and then to each of one pair a small amount of sesame oil containing some free cholesterol, and to each of the other pair, some sesame oil containing cholesterol oleate, and, in some cases, 1 cc. of dog bile, were added. One of each pair was then mixed with alcohol-ether, and the

other, after the addition of a few drops of toluene, incubated from 1 to 6 days, with occasional shaking, and then extracted with alcohol-ether and boiling alcohol. The ether extract finally obtained was divided into two parts as usual, and the part intended for determination of total cholesterol was first titrated in alcohol solution with alcoholic KOH. In each case, as will be seen, there was quite a marked increase in fatty acids, an evidence of good lipase action. If the neutral fat-splitting enzymes could also split cholesterol esters, such action should be apparent here. On the other hand, with the gradual formation of fatty acids, there was abundant opportunity for esterification of the free cholesterol, if such a process could take place under these conditions.

Experiment 15. Pancreas plus Bile.—5 gm. portions of pancreas were used, and 0.4 cc. of sesame oil with, respectively, 0.01 and 0.013 gm. of cholesterol and cholesterol oleate. Incubated 24 hours.

	Titration.	Cholesterol.		F: T.
		Free.	Combined.	
	cc.	*gm.*	*gm.*	
Free cholesterol 0.01 gm., fresh.....	3.0	0.0115	0.0007	94
" " 0.01 " incubated....	14.0	0.0042	0.0042	50
Cholesterol oleate 0.013 gm., fresh.....	3.9	0.0074	0.0047	61
" " 0.013 " incubated.	3.7	0.0015	0.0017	47

Experiment 16. Pancreas plus Bile.—5 gm. portions of pancreas were used, and to each of one pair was added 0.02 gm. of cholesterol in 0.8 cc. of sesame oil, and to the other about 0.04 gm. of cholesterol oleate in 1.0 cc. of sesame oil. Incubated 24 hours.

	Titration.	Cholesterol.		F: T.
		Free.	Combined.	
	cc.	*gm.*	*gm.*	
Free cholesterol 0.02 gm., fresh.....	3.8	0.0212	0.0006	97
" " 0.02 " incubated...	14.8	0.0104	0.0102	50
Cholesterol oleate 0.04 gm., fresh.....	3.5	0.0062	0.0058	52
" " 0.04 " incubated.	17.0	0.0032	0.0100	24

Experiment 17. Pancreas plus Bile.—4 gm. portions were used, and to each of one pair was added 0.02 gm. of cholesterol in 0.8 cc. of sesame oil, and to the other, 0.03 gm. of cholesterol oleate in 0.8 cc. of sesame oil. Incubated 6 days.

	Titration.	Cholesterol.		F: T.
		Free.	Combined.	
	cc.	*gm.*	*gm.*	
Free cholesterol 0.02 gm., fresh.....	Lost.	0.0282	0.0008	97
" " 0.02 " incubated...	10.0	0.0165	0.0120	58
Cholesterol oleate 0.03 gm., fresh.....	Lost.	0.0123	0.0136	47
" " 0.03 " incubated.	10.8	0.0090	0.0131	41

Experiment 18. Pancreas Alone.—4 gm. portions of pancreas were used. To each was added 0.02 gm. of cholesterol in 0.08 cc. of sesame oil. Incubated 24 hours.

	Titration.	Cholesterol.		F: T.
		Free.	Combined.	
	cc.	*gm.*	*gm.*	
Free cholesterol 0.02 gm., fresh	4.6	0.0270	0.0000	100
" " 0.02 " incubated...	13.4	0.0210	0.0109	66

Experiment 19. Intestinal Mucosa plus Bile.—5 gm. portions were used. To each was added 1 cc. of dog bile, and to each of one pair, a solution of 0.02 gm. of cholesterol in 0.8 cc. of sesame oil, and to each of the other two, 0.03 gm. of cholesterol oleate in sesame oil. Incubated 24 hours.

	Titration.	Cholesterol.		F: T.
		Free.	Combined.	
	cc.	*gm.*	*gm.*	
Free cholesterol 0.02 gm., fresh.....	1.0	0.0284	0.0022	93
" " 0.02 " incubated...	13.2	0.0270	0.0030	90
Cholesterol oleate 0.03 gm., fresh.....	1.0	0.0136	0.0162	47
" " 0.03 " incubated.	11.4	0.0130	0.0142	48

In none of these experiments was there any indication of a hydrolytic effect on the cholesterol oleate, in spite of the lipase action which occurred. On the other hand, in all experiments in which the pancreas was used, a rather striking esterification

of the free cholesterol occurred. This was greatest in the experiments in which bile was used, but perfectly definite, also, in the fourth, in which there was only pancreas. In Experiment 1, the decreased amount of cholesterol found in the incubated samples is doubtless due to incomplete extraction. In the experiment with intestinal mucosa, there was no change demonstrable in either the free cholesterol or the oleate.

A synthetic action of this kind is not the rule for enzymes. True, a reversible effect can usually be demonstrated for most enzymes when conditions are carefully controlled. Here, however, the reaction apparently does not reach an equilibrium unless the greater part of the cholesterol has been esterified, since the samples to which cholesterol oleate had been added, and which contained only a relatively small amount of free cholesterol, also gave evidence of an esterification. On the other hand, since an enzyme is a catalytic agent which hastens a reaction that would take place slowly under ordinary conditions, it is not so unlikely that this should be a true enzyme action. Free cholesterol, as is well known, possesses strong inhibiting powers toward hemolytic substances of various kinds. It is not impossible that one of its functions is to combine with and render nontoxic, fatty acids which may become liberated in the body. Apparently it will combine very slowly with fatty acids set free in the brain after death.[9, 10] A raw egg mixed with water, and allowed to autolyze with tricresol and toluene for 6 months showed only 62 per cent of its total cholesterol to be free, as against about 90 per cent at the beginning. Seemingly there is a tendency for free cholesterol to unite slowly with fatty acids to form esters. It is quite conceivable that a body enzyme should hasten such a synthesis.

It is, of course, absolutely essential to show that this esterification did not take place during the extraction and analysis, from heating. The esters are prepared artificially by heating together the cholesterol and an excess of fatty acid to 200°. Possibly the evaporation on the water bath might have been enough to cause the change. Fortunately, the intestinal mucosa experiment, run parallel with those of the pancreas, acts as an

[9] Mair, W., *J. Path. and Bact.*, 1913–14, xviii, 179.

[10] Lapworth, A., and Royle, F. A., *J. Path. and Bact.*, 1914–15, xix, 474.

excellent control. In addition, the effect of evaporating a mixture of oil, fatty acid, and free cholesterol on the water bath was tried. About 0.01 gm. of cholesterol, 0.4 cc. of sesame oil, and 0.4 cc. of fatty acid from saponification of sesame oil were mixed with about 25 cc. of alcohol and 1 or 2 cc. of water, and evaporated down on a water bath. The process was then repeated and the residue allowed to heat for several minutes to 100° after the water and alcohol had evaporated. Analysis of this mixture and of a duplicate unheated mixture showed 0.0130 gm. of free cholesterol before heating, and 0.0131 gm. after heating. In other words, no esters had been formed.

As a further precaution to prevent any possibility of the esterification taking place from heating, the extraction of Experiments 3 and 4 was carried out with as little heating as possible, the extracts concentrated at room temperature before an electric fan, taken up in water, extracted by ether, the ether evaporated until the temperature rose to 45–50°, and then the fatty acids present accurately neutralized with alcoholic KOH, using phenolphthalein as indicator. The solution, after diluting with water, was again extracted with ether, and this solution, free from fatty acids, was analyzed as usual. No difference in results is apparent between these two experiments and the others.

From these experiments it seems fair to conclude that there is in the pancreas some substance which accelerates the formation of cholesterol esters and that possibly the bile increases this effect. It may well be that this action of the pancreas is of fundamental importance in the absorption of cholesterol and that here is to be found the explanation for the failure of cholesterol to be absorbed into the chyle after ligation of the pancreatic ducts.

Influence of the Intestinal Mucosa on Cholesterol Absorption.

In order to study the possible effect of the intestinal mucosa on the absorption of cholesterol, two methods were utilized. The first, an experiment *in vitro* with ground mucosa, already described, gave no evidence of any activity. The second was quite definite in its results. Portions of intestinal mucosa were analyzed during fasting of 24 hours' duration, and during the

active absorption of cholesterol or its esters. It was thought that with the rather considerable amount of fat present in the cells and lymph spaces of the mucosa, enough absorbed cholesterol might be present to be recognizable on analysis. In such experiments, digestion was allowed to proceed for 3 hours, and then the dogs were quickly killed by an intravenous injection of chloroform, the intestine was removed, opened, and washed as free as possible from adherent bile-stained mucus, and the mucosa scraped off with a dull knife. In the same way the material was obtained from dogs which had first been fasted for 24 hours.

The mucosa used for analysis was not carefully weighed out, and the figures are therefore given in grams of cholesterol found, rather than in percentage, and represent portions of mucosa of about 5 gm. weight.

	Duodenum.			Jejunum.			Colon.		
	Free.	Ester.	C:T.	Free.	Ester.	C:T.	Free.	Ester.	C:T.
	gm.	*gm.*		*gm.*	*gm.*		*gm.*	*gm.*	
24 hrs. fasting.....	0.0109*	None.	—						
24 " "	0.0092*	0.0006	6						
24 " "	0.0131	None.	—	0.0098	None.	—	0.0116	0.0006	4.9
Fed three eggs.....	0.0101	0.0021	17						
Fed 300 cc. of milk with 2 gm. of cholesterol in 8 cc. of cottonseed oil.	0.0111	0.0017	13						
Fed three eggs.....	0.0194	0.0061	24	0.0215	0.0064	23	0.0131	0.0014	9.7
Fed two eggs.....	0.0079	0.0014	15	0.0105	0.0012	10	0.0071	0.0002	2.7

* A mixture of duodenum and jejunum mucosa analyzed.

In all the dogs which had been fasted, as well as in the mucosa of the large intestine of the others, there is comparatively little combined cholesterol, in some cases none, and in the others very little. On the other hand, the mucosa of the small intestine of all the dogs which had been fed showed considerably more cholesterol, the amounts ranging from 10 to 23 per cent of the total.

Evidently the cholesterol which is absorbed from the intestine has already been largely esterified by the time it reaches, or at least before it leaves, the lymphatics of the intestinal mucosa.

This fact of itself does not show whether the esters were formed in the intestinal canal and absorbed as such, or whether they were synthesized by the cells of the intestinal mucosa. Taken in connection with the work *in vitro* on the pancreas and intestinal mucosa, however, it seems very probable that the change takes place slowly in the intestine, and that an absorption of the esters takes place as rapidly as they are formed.

No striking difference in the direction of a higher percentage of free cholesterol is apparent in these experiments in which cholesterol was fed. Such an increase, if it existed, could not be recognized with any certainty even by the average results of a considerable number of experiments, for it evidently could not be very great, and there seems to be considerable individual variation in the normal cholesterol content of the intestinal mucosa.

Influence of the Mesenteric Lymph Nodes on Cholesterol Absorption.

No satisfactory experiment could be devised to show a possible effect of the mesenteric lymph nodes on the absorbed cholesterol. It was first attempted to destroy the lymphoid tissue of the body by massive doses of x-ray. A rather small dog was exposed to the rays for about half an hour each day for 5 successive days, during which time the small lymphocytes in the blood fell from 26 to 3 per cent.[11] A thoracic duct fistula was then made, and an analysis of the chyle obtained before and after feeding eggs showed a perfectly normal absorption. After completing the experiment the dog was killed, and the mesenteric lymph nodes, the thymus, and the spleen were examined. The thymus was extremely atrophic, although the dog was quite young. The spleen was rather small and firm, but lymphoid follicles were visible in the gross. The lymph nodes were normal in the gross, but microscopically the germinal centers were somewhat small, and the pulp seemed to contain much less than the usual number of lymphoid cells. But the destruction was by no means sufficient to make it unlikely that they would exercise a normal function on the fat and lipoids passing through them in the chyle.

An attempt to demonstrate the presence of an enzyme in the gland by a technique similar to that used in the case of the pan-

[11] I am indebted to Dr. M. J. Sittenfield for raying the animal for me.

creas and the intestinal mucosa also gave negative results. Fatty acids were produced by the lipase of the glands, and the experiments may thus also serve as controls on the earlier experiments with pancreas.

	Titration.	Free.	Combined.	F: T.
	cc.	*gm.*	*gm.*	
Free cholesterol, fresh.....	0.9	0.0163	0.0002	99
" " incubated.....	5.0	0.0136	0.0006	96
Cholesterol oleate, fresh.....	0.5	0.0047	0.0037	56
" " incubated.....	1.3	0.0036	0.0028	56

In none of the experiments is there any indication either of a saponification of the esters, or of an esterification of the free cholesterol. The lower values found in the fourth are doubtless due to incomplete extraction, for here again it was attempted to avoid an accidental esterification of cholesterol through heating with the fatty acids formed, by the procedure previously described of extracting with no more heat than necessary, and subsequently evaporating before an electric fan, instead of distilling, and neutralizing the fatty acids present.

DISCUSSION.

Of the experimental data here presented there are only a few facts which stand out as definite. First, the bile, and even to a greater extent the pancreatic secretions, seem intimately connected with cholesterol absorption, indeed to a degree somewhat out of proportion to their influence on neutral fat absorption. By experiments *in vitro* it was shown that free cholesterol, in the presence of fatty acids and a suspension of pancreas, undergoes esterification, and from control experiments it seems most likely that this is a real action by the pancreas. In such experiments the addition of a little bile seems to make the change somewhat greater, but not enough experiments of this kind were done to make any generalizations.

By analysis of the intestinal mucosa during starvation and after feeding it was shown that an esterification of at least a large part of the absorbed cholesterol had already taken place.

Since no effect of the mucosa could be demonstrated *in vitro*, it is at least possible that the esterification may take place in the lumen of the intestine under the influence of the pancreatic juice, and that the esters may be absorbed as rapidly as formed.

Again, no evidence that the mesenteric lymph nodes take any part in cholesterol absorption could be obtained. The evidence of their part in neutral fat absorption is, as far as I am aware, based on morphology and differential fat staining, although lymphoid tissue undoubtedly contains an active lipase.

One fact which stands out prominently is the resistance of cholesterol esters to saponification by ordinary active lipases. In no case has there been the slightest evidence of splitting, although neutral fat in the same mixture was easily saponified.

Taken altogether the data here presented by no means explain the whole process of cholesterol absorption. The peculiar effect of the bile in altering the proportion of free cholesterol absorbed is entirely unexplained. More important yet, while the origin of the cholesterol esters seen in the chyle of normal animals after feeding cholesterol-rich meals has been at least in part explained, the increase in free cholesterol and the mechanism which regulates the proportion of the two and keeps it constant has not been cleared up at all. However, the inherent difficulties encountered in carrying out the work make it appear quite useless to seek a more complete understanding of the process by means of the methods at present available.

I am indebted to Dr. W. G. MacCallum and Dr. A. M. Pappenheimer for assistance and suggestions in planning and carrying out the experiments, and to Dr. L. B. Mendel for helpful criticisms in the preparation of the paper.

PANCREATIC DIABETES IN THE DOG.

I. THE INFLUENCE OF ALKALI AND ACID UPON THE GLYCOSURIA AND HYPERGLYCEMIA.

By J. R. MURLIN and B. KRAMER.

(From the Physiological Laboratory, Cornell University Medical College, New York City.)

(Received for publication, September 29, 1916.)

INTRODUCTION.

While controlling the influence of Knowlton and Starling's extract on the metabolism of depancreatized dogs, the writers reported (1) the observation that in a completely depancreatized dog the intravenous injection of 150 cc. of a 1 per cent solution of sodium carbonate in Ringer's solution lowered the G: N ratio from 3.07 to 0.88 within less than 3 hours after the injection was discontinued. A glance at Table I, repeated from the previous paper, shows that the fall in the ratio began at once but within the first 2 hours was due to a rise in the nitrogen output. During the second 2 hours the nitrogen excretion reached its previous level, while the sugar elimination dropped from 0.823 to 0.233 gm. per hour. The sugar per hour then began to rise, but even after 6 hours it had risen to but little more than half of what it was in the period preceding the injection.

TABLE I.

Date.	Time.	Total glucose.	Total nitrogen.	Glucose per hr.	Nitrogen per hr.	G: N.	Volume of urine.
1913		*gm.*	*gm.*	*gm.*	*gm.*		*cc.*
Apr. 24	2.19–4.19 p.m.	1.647	0.519	0.823	0.269	3.07	22
	4.26–5.15 "	150 cc. of 1 per cent Na_2CO_3 in Ringer's solution intravenously.					
	4.19–6.19 "	1.647	0.644	0.823	0.322	2.55	60
	6.19–8.19 "	0.466	0.524	0.233	0.262	0.88	24
" 25	1.19 a.m.	2.413	1.475	0.480	0.295	1.62	90

A search of the literature revealed the fact that in 1910 Pavy and Godden (2) administered a 3 per cent solution of anhydrous sodium carbonate

to cats in which they had induced glycosuria by the administration of chloroform, and observed a fall in the intensity of the glycosuria within 15 minutes after the intravenous injection was begun, the urine becoming sugar-free at the end of 30 minutes. Pavy and Bywaters (3) had previously found that the postmortem production of sugar in the liver may be virtually checked by the influence of sodium carbonate injected in a 5 per cent solution (and even less strength) into the portal system of the living animal. On the other hand, the addition of rising quantities of acetic acid to the liver thus treated brought about an increase in enzymic power, and hence increased glycogenolysis, until a certain optimum was reached. They assumed that the inhalation of chloroform produced a condition of acidosis which led to hyperglycogenolysis and ultimately to glycosuria. The authors referred to seem to have studied the subject no further. They did not attempt to show whether sodium carbonate would exert the same effect in other forms of glycosuria. More recently Elias and Kolb (4) studied the influence of sodium carbonate upon glycosuria, hyperglycemia, and upon sugar tolerance in "hunger diabetes." They conclude from their observations that this alkali reduces the glycosuria and glycemia by influencing the intermediary metabolism of carbohydrates in the liver.

These facts clearly suggest the possibility that administration of alkali to diabetics may be of importance in more ways than one. The express object of alkali therapy is control of the acidosis, but it is possible that in so restoring the balance of ions in the tissues, the glycogenic functions and even the combustion of glucose may be assisted. The chance observation made while controlling the effect of Knowlton and Starling's extract of pancreas (5) has been followed up now for more than 3 years in this laboratory and we have accumulated much evidence that the regulation of acid and basic radicals and possibly the regulation of the kations amongst themselves is an important factor in preserving the capacity to oxidize glucose. A full discussion of the bearing of these facts will be deferred until the facts themselves are in evidence.

In this paper will be presented the facts first developed in our study of the influence of sodium carbonate upon the metabolism of the dog after pancreatectomy, as far as they relate to urine and blood. Coincidentally with these observations, some preliminary data were obtained regarding the respiratory metabolism, but it will be more convenient to present the evidence from this quarter *in extenso* separately.

Methods of Experimentation.

Female dogs large enough to permit of passage of a catheter with the help of a speculum have been used exclusively and the urine has in nearly every instance been so separated in definite periods. The bladder was always irrigated with saturated boric acid solution at the close of each washing. The urines have been most carefully preserved, as a rule with 10 per cent thymol in toluene (though in some instances with concentrated sulfuric acid) and refrigeration to 5°C.

Blood has been drawn, both with the needle by venapuncture, and after insertion of a cannula under local anesthesia. We prefer the latter method and in our later experiments have followed it almost exclusively because of the greater precision with which a sample can be taken at a desired time. No difficulty has been experienced on account of the "emotional glycosuria" of Cannon, Shohl, and Wright (6). However, while Scott (7), Seelig (8), and Rolly and Oppermann (9) do not believe that the dog is subject to emotional *glycosuria*, Loewy and Rosenberg (10) have shown that pain or fright incident to drawing blood may increase the *glycemia* to quite abnormal figures, and Shaffer (11) presents evidence to the same effect. We have, therefore, always exercised great care to avoid the causation of pain and to keep the animal perfectly quiet for some time preceding the opening of a vessel.

In most instances the blood has been drawn into a Brodie pipette by displacement of mercury, the blood mixing as it enters with 0.3 cc. of leech extract (hirudin). We agree with Epstein and Baehr (12) as to the importance in comparative analyses of knowing whether the blood has itself undergone a change of concentration and, as a means of control, we have made many determinations of the total specific gravity by first measuring accurately 1 or 2 cc. of blood into a weighed flask and immediately weighing the sample. Satisfactory duplicates of the specific gravity have nearly always been obtained in this way.

There are obvious advantages in the use of the specific gravity rather than the ratio of blood plasma to corpuscles as determined by means of the hematocrit. Hamburger and others have shown that the blood corpuscles change considerably in volume with changes in the concentration of the plasma and, in fact, the most common use of the hematocrit is for the demonstration of such changes in volume of the corpuscles rather than for demonstration of a change in concentration of plasma (13). Moreover, since the blood sugar is found in the corpuscles as well as in the plasma, it is difficult to see how a knowledge of the relation of plasma to corpuscles helps to give the correct percentage of blood sugar. A blood count would be a better index.

The method of Lewis and Benedict (14), either as originally reported, or as published in final form, has been used throughout for the blood sugar, and the method of Benedict for the urinary sugar.

Control of the Effect of Alkali on the Sugar in the Urine.

Several observers, notably Bendix and Bickel (15) and Mathews (16), have noted the spontaneous disappearance of sugar in alkaline solution. Although the quantity destroyed in this way is small, it might be sufficient in urines which had been rendered strongly alkaline by administration of sodium carbonate to account for a portion, at least, of the apparent retention. It was therefore necessary to assure ourselves that this possible error was at least not very large.

A specimen of urine was obtained by catheter from a completely depancreatized dog, its volume made up to 500 cc., and the entire quantity divided into two equal parts. The sugar in one portion was determined at once and found to be 1.49 gm. The second portion was made alkaline with sodium bicarbonate, placed in the incubator, and kept there at a temperature of 36°C. for 7 hours. At the end of this time the urine was found to contain 1.527 gm. of dextrose.

A second urine, faintly alkaline and containing 30 gm. of glucose for the 24 hours, was diluted to proper strength for titration by Benedict's sugar method. The titration for 25 cc. of the copper solution was 10.2 and 10.1 cc. After standing 6 hours in the incubator at 36° and 18 hours at room temperature the titration next day was 10.0 and 10.1 cc. Still another portion, made more strongly alkaline with sodium carbonate and kept beside the first control, gave a titration next day of 9.9 and 10.0 cc.

A similar control with sodium carbonate was performed by Elias and Kolb (4) in their work upon hunger diabetes.

Another factor that must be taken into account is the possible formation of complex condensation products of glucose, such as those found by Levene and Meyer (17) to be produced by the action of mixed extracts of pancreas and muscle upon sugar solutions in alkaline medium. To control this factor, a urine which had been rendered sugar-free by the administration of sodium carbonate to the animal from which it had been obtained, was boiled with a reflux condenser for 1 hour after acidifying with concentrated hydrochloric acid; it was then made alkaline with sodium carbonate and was tested for sugar with Benedict's qualitative sugar reagent, with negative results.

The Importance of Body Temperature.

Opinion is divided as to the influence which infection may have on the course of the diabetes produced by total pancreatectomy. Allen (18) reports many instances of peritonitis following partial pancreatectomy in which the infection appeared to be without specific influence on the course of the disease. Our own experience with partial pancreatectomy is very limited as yet, but we have seen instances of rapid death with high fever (40° and upward) following the total operation in which there seemed to be

no other cause for the failure of glycosuria, and we have gained the impression that a serious infection with rise of body temperature is of the greatest moment. Accordingly, we have from the start kept a daily record of body temperature and are satisfied that we have spared ourselves much time and trouble by avoiding the use of animals with a high temperature.

A. The Administration of Alkali by Mouth.

It seemed strange, in view of the marked effect of alkali on the elimination of glucose by the depancreatized dog, that such effects had not long ago been observed after the administration of alkali to diabetic patients. Practically every diabetic patient in routine treatment has received alkali by mouth. It is commonly given in the form of sodium bicarbonate. Only in the face of threatening coma is the more strongly alkaline normal salt usually given or any alkali administered by vein. Patients of intelligence vouch for a marked improvement in their subjective feelings from the use of sodium bicarbonate and some claim the power to discern its need by an acid taste in the mouth or by a general feeling of depression. Why, then, has not some improvement in the glycosuria itself been observed? It is our belief that much of the benefit ascribed to the "oat cure" or other specific carbohydrate "cures" is really to be credited to the large amounts of sodium bicarbonate given at the same time. Weiland (19) says: "Wir haben zwar in allen Fällen dieser Art vom Hafer Gebrauch gemacht, ihn aber stets mit ausgiebiger Alkalizufuhr . . . . so dass eine eindeutige Haferwirkung nicht zu erkennen war. Viel grössere Bedeutung kommt hier der Zufuhr von Alkali zu." Possibly, oats and $NaHCO_3$ together supply the proper ratio of kations. In many of Weiland's cases the urine became sugar-free while large doses of alkali were being given. This has been seen by many other observers, but Weiland is the first, as far as we are aware, to recognize the alkali as a *possible* factor in diminishing the glycosuria. Recently, Underhill has reported a case that became sugar-free after the daily ingestion of $NaHCO_3$ in Vichy water had reached 120 gm. and was then kept in this state by smaller doses diminishing to 40 or 50 gm. (27).

As far as we can discover, nobody has attempted treatment of

the dog made diabetic by pancreatectomy with alkali, after the manner of its use with patients. The question naturally arises whether the dog reacts differently in this respect from the human subject.

The experiments reported below show that in many instances there may be little or no effect from bicarbonate.

TABLE II.

Contrast between per Os and Intravenous Administration of Sodium Carbonate. Dog 3. Weight 7.95 Kg. Pancreatectomy July 18, 1914, 10 a.m.

Date.	Time.	Total G.	Total N.	G per hr.	N per hr.	G : N.	Blood sugar.	Sp. gr.	Temperature.	Remarks.
1914									°C.	
July 21	2.20 p.m.	2.55	0.952			2.73				
	2.30 "						0.292	1.068	38.3	17.89 gm. glucose given at 2.40 p.m.
" 22	10.15 a.m.	35.26	5.848	0.80	0.265	3.05				17.73 gm. recovered.
	11.00 "									5 gm. Na_2CO_3 by stomach tube.
	4.45 p.m.	7.05	2.00	1.08	0.307	3.52				
	4.50 "						0.198	1.078	38.2	
" 23	10.38 a.m.	11.244	5.92			1.89				
	2.08 p.m.						0.288	1.059		
	2.10–2.35 p.m.									Infusion 200 cc. distilled water + 1.5 gm. Na_2CO_3.
	5.45 p.m.	1.93	1.23	0.48	0.310	1.56				
	6.12 "						0.292	1.060		
" 24	Autopsy.	No pancreatic tissue found.								

Dog 3, whose pancreas was removed on July 18, 1914, at 10 a.m., had on July 21 a perfect Minkowski ratio and eliminated between 2.30 p.m. of this day and 10.15 a.m. the next day 17.73 gm. out of 17.89 gm. of glucose administered by mouth. The intolerance for carbohydrate was therefore complete. At 11 a.m. of the 22nd 5 gm. of sodium carbonate were given by stomach tube and at 4.45 p.m. urine obtained by catheter showed an increase

in the hourly elimination of both nitrogen and glucose, but the increase in the glucose was greater than that of the nitrogen, the result being, therefore, a higher G: N ratio. The blood sugar fell coincidentally with the rise in glucose excretion. At 10.38 a.m. on the 23rd the G: N was 1.89. The hourly elimination could not be computed. In the afternoon between 2.10 and 2.35, an infusion of 1.5 gm. of sodium carbonate in 200 cc. of distilled water was given. The G: N ratio fell to 1.56, the blood sugar and specific gravity changing only slightly. The normal salt, with its higher alkalinity, produced a prompt fall in the elimination of glucose when given by vein, but the effect was much delayed when given by mouth.

TABLE III.

Bicarbonate of Sodium and Potassium. Dog 8. Weight 8 Kg. Pancreatectomy August 26, 1914.

Date.	Time.	Total G.	Total N.	G per hr.	N per hr.	G: N.	Temperature.	Remarks.
1914							°C.	
Sept. 1	4 p.m.							10 gm. $NaHCO_3$. in 400 cc. water; all retained.
	6 "							100 gm. meat.
" 2	Cage urine.	4.84	1.764			2.74	38.5	
	Catheter 12 m.	5.85	2.548			2.29*	38.9	
	" 4 p.m.	1.10	0.728			1.51		500 cc. 1 per cent $KHCO_3$ at 4.10. 100 gm. meat at 5.30.
" 3	Cage urine.	6.33	1.932			3.27		
	Catheter 1 p.m.	5.18	2.478			2.09**		
	" 6 "							20 gm. $NaHCO_3$ up to 4 p.m. 200 gm. meat at 6 p.m.
	" 8 "	1.527	0.469	0.763	0.234	3.25**		
" 4	1 a.m.	7.780	4.51	0.518	0.300	1.72**	38.9	
	Catheter 11 "							
	" 1 p.m.	6.501	0.392	0.250	0.196	1.27*	39.1	
	Date of death lost. No pancreatic tissue found.							

* Urine acid.

** Urine alkaline.

Dog 8, depancreatized on Aug. 26, 1914, received on Sept. 1 at 4 p.m. 10 gm. of sodium bicarbonate in 400 cc. of water. At 6 p.m. 100 gm. of meat were eaten. The next morning the cage urine showed a G: N ratio of 2.74. At 12 m. urine from the bladder contained less glucose in proportion to the nitrogen, and the urine was acid, showing that the alkali had by this time been entirely eliminated. At 4 p.m., without further treatment, the ratio had fallen still further. Bicarbonate of potassium was then given in 1 per cent solution and the same quantity of meat was eaten. The next morning the ratio of glucose to nitrogen excreted in the cage urine was 3.27, while at 1 p.m. it had returned again nearly to the level it had shown at a corresponding time the day before when bicarbonate of sodium had been given. In brief, the two salts had produced nearly identical (if any) results. As will be shown in a later paper, the G: N of the depancreatized dog varies with the time after feeding, being above the Minkowski ratio in the early hours and below it in the later hours, but making an average for the 24 hours very close to the average given by Minkowski as characteristic for the meat-fed dog. Reilly, Nolan, and Lusk (20) have shown that the same is true, though the difference is much less striking, in the phlorhizinized dog; *i.e.*, the glucose derivable from protein is excreted more rapidly than the nitrogen. This is doubtless the meaning of the downward tendency of the ratio seen here after each feeding. Possibly, there was at the same time some delay in the gastric digestion caused by the presence of the alkali. On the evening of Sept. 3 the experiment with sodium bicarbonate was repeated, using, however, double the amount of alkali, and 2 hours later, double the amount of meat. At 2 and 7 hours later, first a high and then a low G: N was again found. At 1 p.m. the urine was acid, showing that the alkali had all escaped; but the ratio was still lower. At first sight, these observations appear to prove that the bicarbonates have a delayed effect in bringing the ratio down. There is reason, however, as we have seen, to believe that the ratio would have been somewhat lower at this time if the alkali had not been given.

Moreover, as the following experiment proves, bicarbonate administered to a fasting depancreatized dog may have the effect of causing sugar to reappear in the urine after it had entirely vanished.

TABLE IV.

*The Influence of Bicarbonate on a Fasting Depancreatized Dog. Dog 7. Weight 4 Kg. Pancreatectomy August 19, 1914. Fasting since August 15.**

Date.	Time.	Total G.	Total N.	G:N.	Temperature.	Reaction.	Remarks.
1914					°C.		
Aug. 21	Catheter 1.20–4.20 p.m.	0.0	0.525		38.7	Alkaline.	Refused all food.
	4.30 "						350 cc. 2 per cent $NaHCO_3$ by stomach tube.
	Specimen 6.30 "	0.695	0.266	2.61	38.7		Refused all food.
	" 7.05 "	0.631	0.283	2.23			
" 22	" 9.00 a.m.	1.238	0.794	1.56			
	Voided 10.00 "	0.185	0.595	0.3			Refused food.
	Catheter 11.30 "	0.0	0.192				
	12.00 m.						4 gm. $NaHCO_3$ in 300 cc. water by stomach tube.
	Voided 1.30 p.m.	0.185					
" 23		0.0					Refused food.
" 24	11.30 a.m.	0.0			39.3		" "
	3.15 p.m.						17.89 gm. glucose given in 300 cc. water; none vomited.
" 25	3.10 "	13.684			38.7		4.206 gm. retained.
" 28	Respiratory experiment. R. Q. after 20 gm. glucose, 0.80 and 0.79						
Sept. 2	Dog killed. No pancreatic tissue found. Neighboring tissues sectioned; no pancreatic tissue. Urine had been free of sugar to end.						

* Pancreas was sclerotic and brittle; very easily removed.

Dog 7 had been in the laboratory for 4 days previous to operation and had refused all food during this time. The pancreas at operation was found to be quite sclerotic and brittle but was easily removed. 2 days afterward the urine, to our surprise, was found at 4.30 p.m. to be sugar-free. The dog meantime had eaten nothing. At 4.30, 350 cc. of 2 per cent solution of sodium bicarbonate were given by stomach tube and all was retained.

At 6.30, a G: N ratio nearly approaching the typical for meat-fed depancreatized dogs was obtained and the sugar continued in the urine until next day at 10 a.m. When, at 11.30, the urine was again found sugar-free, more bicarbonate was given and once more a small amount of sugar came out. The next 2 days, without any treatment, the urine continued sugar-free and on the 24th 20 gm. of commercial glucose, containing 17.89 gm. of actual glucose, by titration, were given at 3.15 p.m. 24 hours later 13.68 gm. had been excreted. This indicated a moderate tolerance, and judging from the results of the respiration experiment performed on the 28th, it is possible that some of the sugar was burned. *Autopsy revealed no pancreatic tissue although all fragments of tissue suspected of containing pancreas were sectioned.* The dog also was not pregnant unless in the very early stages when gross examination of the uterus would reveal nothing.

This interesting case will be alluded to in the third paper but is mentioned here for the purpose of showing that sodium bicarbonate, with its very slight alkalinity, may not only produce no reduction in the excretion of sugar, as seen in the last experiment, but may even call out sugar otherwise absent from the urine.

Dog 5, deprived of the pancreas on July 29, 1914, showed, 2 days later, a G: N ratio indicating a nearly total diabetes. By the administration of 7.5 gm. of the normal anhydrous salt in 1 per cent solution the urine was rendered sugar-free and this continued for 4 days under continued treatment with the alkali, notwithstanding the ingestion of 200 gm. of meat on one of these days. The blood sugar fell at first, then returned to 0.15 per cent, at which point it remained when last examined. This is a rather low percentage of blood sugar for total diabetes and, in order to make certain that the diabetes was total, it was necessary to make use of the respiratory quotient. Two periods on Aug. 4 were sufficient to establish the fact that there was no capacity on the part of the organism, even with the aid of the alkali administered by mouth, to oxidize glucose. After administration of 20 gm. of the sugar, with more carbonate, there was a slight rise in the R. Q. in the 2nd and 3rd hours, but it had completely subsided in the 4th. Many such experiments have been done in this same manner. The R. Q. and heat production have been

TABLE V.

Dog 5. Weight 7.7 Kg. Pancreatectomy July 29, 1914, 2 p.m.

Date.	Time.	Temperature.	Glucose per hr.	Nitrogen per hr.	G:N.	CO_2	O_2	R. Q.	Blood sugar.	Sp. gr.	Remarks.
1914		*°C.*				*liters*	*liters*		*per cent*		
July 31	2.18		0.445	0.189	2.34				0.164	1.058	250 cc. 1 per cent Na_2CO_3
	5.10		0.383	0.219	1.74						500 " 1 " " "
Aug. 1		38.1	0.0						0.118	1.055	500 " 1 " " "
" 2			0.0								
" 3			0.0								200 gm. meat + 0.5 per cent Na_2CO_3.
" 4	10.30	38.2	0.0						0.153	1.060	300 cc. 1 per cent Na_2CO_3 by stomach tube. Vomited.
	Respiratory experiment.										
	12.15–1.15					2.916	4.439	0.66			300 cc. 0.5 per cent Na_2CO_3 subcutaneously.
	3.09–4.09					2.932	4.259	0.69			
	4.30	38.4	0.0	0.120					0.150	1.060	400 cc. 0.3 per cent Na_2CO_3 subcutaneously. 20 gm. glucose *per os*.
	Respiratory experiment.										
	5.27–6.27					2.926	4.283	0.68			
	6.27–7.27					3.083	4.319	0.71			
	7.27–8.27					2.789	3.863	0.72			
	8.27–9.27					2.919	4.260	0.69			
" 5	Dog dead.	No pancreatic tissue found.									

determined after the administration of carbonate alone and of carbonate with sugar several days after total pancreatectomy, but never have we seen a R. Q. higher than 0.73 *following the administration by mouth* of sodium carbonate with totally depancreatized dogs. As we shall see, the result is different after partial pancreatectomy.

B. The Administration of Alkali by Vein.

Our first positive result was obtained with Ringer's solution made alkaline to the extent of about 1 per cent with sodium carbonate.[1] Returning to this procedure, we have been able to reproduce the first result a number of times. The only failures were traceable to the use of bichloride of mercury in too concentrated a solution as a hand wash at operation. The following experiments are typical of the effects on urinary and blood sugar.

TABLE VI.

Dog 31. Pancreatectomy February 8.

Date.	Weight.	Urine.	Temperature.	Glucose.	N.	G: N.	Blood sugar.	Remarks.
1915	*kg.*		*°C.*				*per cent*	
Feb. 9	13.20	Catheter.	38.6	4.72	1.18	4.00		
		Cage.		17.73	6.02	2.94		
" 10	12.90	Catheter 9.40	37.6	0.50	0.18	2.78		
		12.40		2.18	0.364	5.9	0.40	1.00–1.15, 200 cc. Locke's solution + 1.5 gm. Na_2CO_3 by vein.
		" 3.40	40.2	1.49	0.49	3.04	0.42	
		" 7.00		0.00	0.196	0.0	0.38	
		" 10.00		0.00		0.0		

Dog 31. 2 days after operation 200 cc. of Locke's modification of Ringer's solution (containing, however, no glucose) were infused into the jugular vein. The infusion was stopped at 1.15

[1] The addition of carbonate to Ringer's solution naturally precipitated the calcium, but this does not interfere with its use intravenously.

p.m., and at 3.40—2 hours and 25 minutes later—the sugar had completely disappeared from the urine. It was still absent at 10 p.m.

Three points are of special interest in this experiment: First, the high G: N ratio found at 12.40 p.m. The dog had exhibited the typical Minkowski ratio at 9.40 a.m. and, without having been fed, developed within 3 hours a ratio more than twice as high. The occurrence of high ratios at a corresponding period has been noted several times in the course of the work. An explanation of this peculiar behavior will be given in due time. At the moment it is of interest as showing how differently the depancreatized dog may behave from the dog kept uniformly intoxicated with phlorhizin (21). The second point of special interest is the decrease in nitrogen elimination coincidentally with the cessation of sugar elimination. The nitrogen output per hour falls to about half what it was before the infusion, but follows immediately upon a somewhat heightened excretion. In fact, if the whole elimination for the 6 hours be computed to the hourly basis it is seen that there is but very slight change from the rate at which the kidney was putting forth nitrogen previous to infusion. The more rapid elimination at first is doubtless due to a washing out process brought about by the dilution of the blood, and the slower elimination later is, in all probability, merely the natural compensation of this. Without the preliminary washing out one would be inclined to ascribe the whole change, as regards both sugar and nitrogen, to an effect on the kidney. Instead of a sweeping out of sugar, however, we see a gradual fall followed by total disappearance. The third point of interest is the behavior of the blood sugar. Were the effect of the infusion wholly a renal effect, one would expect an increase in total blood sugar[2] as the urinary sugar fell off, and there is indeed some indication of this in the slightly augmented percentage at the 3 hour period. Two facts prevent a definite conclusion from this experiment: The specific gravity was not determined, hence one cannot say to what extent the blood was diluted at this time; and, second, there is always a possibility that sugar has been withdrawn from the circulation under the influence of the

[2] See Appendix to Table III of the second paper for an instance of augmented blood sugar caused by failure of the kidney to produce urine.

alkali. This factor, as far as it may be related to a form of carbohydrate which can be mobilized again by adrenalin, will be discussed in the second paper.

TABLE VII.

Dog 32. Weight 12.55 Kg. Pancreatectomy February 13.

Date.	Time.	Temperature.	Volume of urine.	G.	N.	G : N.	Blood sugar per 100 cc. blood.	Sp. gr.	Remarks.
1915		°C.	cc.				gm.		
Feb. 16	Catheter 9.40	38.6		1.60	0.50	3.20			
	" 11.40		32	2.31	0.67	3.44			
	" 1.40		36	2.55	0.71	3.59	0.25	1.056	1.40–2.07, infusion of 300 cc. Locke's solution containing 3 gm. Na_2CO_3.
	" 3.40	40.0	100	1.64	0.71	2.31	0.24	1.049	
	" 5.40	38.8	30	0.00	0.46	0.00	0.20	1.043	
	" 7.45		200*	1.67	0.55	3.04	0.19	1.042	

* Dog had a large drink of water at 6 p.m.

Dog 32. On the 3rd day after operation, the G: N ratio in an early morning specimen from the bladder was high enough to indicate total diabetes. Two control periods of urine were obtained and a sample of blood was taken just before the infusion was begun. Within 2 hours the sugar had disappeared from the urine. Remaining absent for a complete 2 hour period, it was present again at the next examination 2 hours later in the same quantity as at the end of the first 2 hours. It is difficult to interpret the behavior of the nitrogen. Taking the figures in the control periods as normal, there appears to be a considerable fall coincident with the disappearance of sugar; but if the figure found at the end of the first 2 hour period after infusion may be interpreted as a rise due to washing out of extractive nitrogen, as was seen in the previous experiment, then the amount recovered at the last examination may be regarded as a return to the normal level which prevailed early in the morning.

Turning to the blood sugar, it is evident that the fall in per-

centage is not so great as would be expected from the change in specific gravity. The first step from 1.056 to 1.049 would be brought about by the addition of water to the extent of 25 per cent of the blood volume; the second drop to 1.043, by the addition of 46 per cent of the blood volume. If no sugar were held back by the kidney, such a dilution would produce a change in percentage from 0.25 to 0.20 per cent and the next step (1.043) would find it at the level of 0.17 per cent. Since only 100 cc. of urine were obtained at the end of the first 2 hour period, it is probable that more than enough water had actually been retained to account for the change in gravity (allowing 600 gm. of blood for a 12 kg. dog).

To produce the gravity found at the next examination, however, would require the retention of 275 cc. of water. It is possible that fluid might be withdrawn from the tissues to produce this result.

Assuming 600 gm. of blood at the beginning, 0.25 per cent of sugar would amount to 1.5 gm. as the total amount in circulation. At the time when the sugar was found entirely absent from the urine, however, there was 0.2 per cent in the blood, and if we assume that at least 275 cc. of water had been added to the circulating fluid (to produce the observed change in gravity), there must have been all told about 1.75 gm. of sugar in circulation, or an increase of 0.25 gm. Since before the infusion sugar was being excreted at the rate of 1.2 gm. per hour, whereas after the infusion only 1.6 gm. were recovered in 4 hours, it is evident that at least 3.2 gm. had been diverted. Only a small part of it can have remained in the circulation.

C. The Influence of Hydrochloric Acid on the G: N Ratio.

In the work previously reported the effect of a single dose of 2 per cent hydrochloric acid by stomach on the glycosuria of the depancreatized dog was noted (22). The rate of glucose elimination was increased for as much as 4 hours, while the rate of nitrogen remained constant. The same may be seen in the following experiments.

Dog 22, bull terrier, female, about 9 months of age, weight 7.9 kg., was depancreatized on Jan. 5, 1915. This dog was used for

the determination of the mineral alkalinity of blood (23) in connection with the respiratory metabolism for several days. A complete Minkowski ratio on a meat diet was obtained on the 3rd day after pancreatectomy (Jan. 8). On this day, however, the dog refused food and, after it was given, vomited the entire meal. On Jan. 9 the following observations were made.

TABLE VIII.

Time.	G.	N.	G : N.	G per hr.	N per hr.	Remarks.
	gm.	*gm.*		*gm.*	*gm.*	
10.15 a.m.	3.97	2.52	1.58			
12.15 p.m.						300 cc. 0.15 per cent HCl by stomach tube.
1.30 "	4.24	1.00	4.24	1.30	0.30	
4.45 "	1.61	0.99	1.63	0.49	0.30	

Dog 27, brindle bull, female, probably about 8 months of age, weight 11.8 kg., was depancreatized on Jan. 29, 1915. On the morning of the 30th, without having eaten any food since operation, the dog showed a G: N ratio of 2.50. On the morning of Feb. 1 the ratio in the cage urine was only 1.78, but the bladder urine at 2.20 p.m. gave a ratio of 2.84. At 2.35, 100 cc. of 0.3 per cent hydrochloric acid were given subcutaneously. The results follow.

TABLE IX.

Date.	Time.	G.	N.	G : N.	G per hr.	N per hr.	Remarks.
1916		*gm.*	*gm.*		*gm.*	*gm.*	
Feb. 1	10 a.m.	23.53	13.25	1.78			
	2.20 p.m.	5.17	1.82	2.84	1.19	0.42	
	2.35 "						100 cc. 0.3 per cent HCl subcutaneously.
	5.20 "	5.84	1.62	3.60	1.94	0.54	
" 2	10.10 a.m.	25.00	8.77	2.83	1.47	0.51	

The results of these two experiments confirm in every particular those previously reported. With the nitrogen remaining constant, the sugar output per hour is increased in one instance about threefold. In the other, the nitrogen level is not so con-

stant—a rise of approximately 30 per cent can be made out—but the glucose rises about 80 per cent in the first 3 hours and evidently continued high for some time thereafter.

Mention should be made at this point of the extreme toxicity of hydrochloric acid for the depancreatized dog. Eppinger (24) has shown that a dog of 6.5 kg. after removal of the pancreas succumbed to a dose of 2.7 gm. of hydrochloric acid, or about 0.4 gm. per kilo, and another of 7.6 kg. was brought to the point of impending death (when the dog was bled for the purposes of blood analysis) by a dose somewhat less than this. On the other hand, a normal dog (25) on a meat diet will withstand a dose of 0.9 gm. of hydrochloric acid per kilo and will neutralize 80 per cent of the acid with ammonia.[3] Confirmation of these observations has been seen in the course of this work.

Dog 25, a young female, weighing 7.4 kg., was depancreatized on Jan. 23, 1915. On the 25th she received 0.6 gm. of hydrochloric acid in 300 cc. of water by stomach tube. Two-thirds of this was vomited a half hour later. The same evening the dog was given a meal of meat and lard but it was vomited during the night. On the 26th at 1 p.m. 0.5 gm. of hydrochloric acid was given in 25 cc. of water subcutaneously. The next day the dog vomited excessively all the morning. At 12.50 p.m. 0.3 gm. more of hydrochloric acid was given in 100 cc. of water intraperitoneally. The dog died in coma at 6 p.m. The total amount of acid given was 1.4 gm., or altogether less than 0.2 gm. per kilo.

SUMMARY.

1. Sodium bicarbonate and potassium bicarbonate administered by stomach tube may be without immediate effect on the glycosuria and the hyperglycemia of the depancreatized dog.

2. A bicarbonate given by mouth to a fasting depancreatized dog may even cause the reappearance of glucose in the urine after it had been "starved out."

3. The normal anhydrous salt of sodium, Na_2CO_3, may, on the contrary, reduce the sugar in the urine materially when given

[3] Schryver (26) has emphasized the importance of ammonia derivable from the products of tryptic digestion as a protection to the liver against the autolytic effects of acids.

by mouth, and when given by vein invariably does so, especially when added to Ringer's or Locke's solution to the amount of about 1 per cent.

4. The blood sugar does not undergo a compensating increase in percentage even when the dilution of the blood is accounted for.

5. Dilute HCl given by mouth or subcutaneously to the depancreatized dog has just the opposite effect of alkali—increasing the sugar in the urine without affecting materially the nitrogen elimination—and without causing any effect on the blood.

BIBLIOGRAPHY.

1. Murlin, J. R., and Kramer, B., *J. Biol. Chem.*, 1913, xv, 365.
2. Pavy, F. W., and Godden, W., *Proc. Physiol. Soc.*, 1911, xliii, p. vii.
3. Pavy, F. W., and Bywaters, H. W., *J. Physiol.*, 1910, xli, 168.
4. Elias, H., and Kolb, L., *Biochem. Z.*, 1913, lii, 331.
5. Knowlton, F. P., and Starling, E. H., *J. Physiol.*, 1912, xlv, 146.
6. Cannon, W. B., Shohl, A. T., and Wright, W. S., *Am. J. Physiol.*, 1911–12, xxix, 280.
7. Scott, E. L., *Am. J. Physiol.*, 1914, xxxiv, 271.
8. Seelig, A., *Arch. exp. Path. u. Pharm.*, 1905, lii, 481.
9. Rolly, F., and Oppermann, F., *Biochem. Z.*, 1913, xlviii, 200.
10. Loewy, A., and Rosenberg, S., *Biochem. Z.*, 1913, lvi, 114.
11. Shaffer, P. A., *J. Biol. Chem.*, 1914, xix, 297.
12. Epstein, A. A., and Baehr, G., *J. Biol. Chem.*, 1916, xxiv, 1.
13. Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 3rd edition, 1911, 74.
14. Lewis, R. C., and Benedict, S. R., *Proc. Soc. Exp. Biol. and Med.*, 1914, xi, 57; *J. Biol. Chem.*, 1915, xx, 61.
15. Bendix, E., and Bickel, A., *Deutsch. med. Woch.*, 1902, xxviii, 3.
16. Mathews, A. P., *J. Biol. Chem.*, 1909, vi, 3.
17. Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1911, ix, 97.
18. Allen, F. M., Glycosuria and Diabetes, Boston, 1913, 492.
19. Weiland, W., *Z. exp. Path. u. Ther.*, 1912–13, xii, 116.
20. Reilly, F. H., Nolan, F. W., and Lusk, G., *Am. J. Physiol.*, 1898, i, 395.
21. See especially the papers of Lusk and of Ringer.
22. Murlin and Kramer, *J. Biol. Chem.*, 1913, xv, 376.
23. See a later paper of this series.
24. Eppinger, H., *Wien. klin. Woch.*, 1906, v, 111.
25. Eppinger, *Z. exp. Path. u. Ther.*, 1906, iii, 530.
26. Schryver, S. B., *Biochem. J.*, 1906, i, 123.
27. Underhill, F. P., *Proc. Soc. Exp. Biol. and Med.*, 1916, xiii, 111.

PANCREATIC DIABETES IN THE DOG.

II. IS THE GLUCOSE RETAINED WHEN SODIUM CARBONATE IS ADMINISTERED TO DEPANCREATIZED DOGS DEPOSITED AS GLYCOGEN?

By B. KRAMER, J. MARKER, and J. R. MURLIN.

(From the Physiological Laboratory of the State University of Iowa, Iowa City, and the Physiological Laboratory, Cornell University Medical College, New York City.)

(Received for publication, September 29, 1916.)

Recent work upon the problems of glycogenesis and glycogenolysis points clearly to the fact that sodium carbonate exerts an inhibitory effect upon those processes which cause a liberation of glycogen from its hypothetical intracellular combination and which bring about a cleavage of the glycogen molecule into glucose. Pavy and Bywaters (1) reported that the injection of acids into the portal vein, ante mortem, increases postmortem glycogenolysis, whereas the injection of sodium carbonate has the opposite effect. As early as 1882 Einhorn, working in Ehrlich's laboratory, showed that, while winter frogs neither lose glycogen from their livers nor form new glycogen when immersed in a plain sugar solution, they do lose their glycogen if the sugar solution is acidulated (2). On the other hand, when such frogs are immersed in a dextrose-sodium carbonate solution, a demonstrable formation of new glycogen from available glucose takes place without the occurrence of glycogenolysis. A diminution in glycogenolysis and even an actual glycogenesis has been observed by Elias (3) in turtle livers perfused with Ringer's solution containing sodium carbonate. Furthermore, Elias and Kolb were able to bring about a considerable loss of glycogen from the livers both of rabbits and dogs by the administration of large doses of 0.1 N hydrochloric acid (4). Macleod and Pearce (5) also have demonstrated the extreme sensitiveness of the glycogenolytic process to the percentage of CO_2 (hydrogen ion concentration) in the blood passing through the liver.

Thus the antagonism of acids and alkalies in accelerating or retarding the processes of glycogenolysis and glycogenesis seems clearly established for normal animals, and one cannot help thinking that the action of sodium carbonate in decreasing the intensity of the glycosuria, as reported in the preceding paper (6), may be due to the fact that it favors the retention of preformed glycogen and brings about a formation of new glycogen from available glucose.

Methods of Experimentation.

Two methods were used in an attempt to demonstrate a retention of glucose in the form of glycogen. First, by direct determination of the glycogen contained in the liver and muscles of a depancreatized dog in which a retention of glucose had been brought about by the administration of sodium carbonate; second, by a method suggested to us by Dr. R. T. Woodyatt, the steps of which were as follows: A completely depancreatized dog was deglycogenized by the repeated subcutaneous injection of small doses of adrenalin, as described by Sansum and Woodyatt (7). The animal was then fed on some glucogenetic material, usually lean meat. Sodium carbonate was administered in repeated doses, either *per os* or intravenously in 1 per cent solution, and after a considerable quantity of glucose had been retained, was again deglycogenized by repeated injections of adrenalin. The elimination of extra sugar was to be taken as proof that the sugar which failed to appear in the urine during the administration of the carbonate had been retained as glycogen. The detailed description of these experiments follows.

Experiment 1 (*Table I*).—The dog was depancreatized in the two stage operation of Minkowski, but following the rapid technique of Hédon. The urine was then collected in definite periods of about 24 hours each and as soon as the G: N ratio reached 2.62, approximately the typical Minkowski ratio, the experiment was begun. The dog was given 2 gm. of sodium carbonate in 1 per cent solution by stomach tube at 10 a.m. and a second dose at 1 p.m. The G: N on the following day fell to 1.90. The total amount of sugar retained was 6.86 gm., the glucose retained thus being the difference between the amount that theoretically should have been eliminated and the amount actually excreted. The carbonate was continued for the next 48 hours in 2 gm. doses twice a day. On the 4th day 300 gm. of lean beef heart were given; on the 5th day, 400 gm., and on

the 6th, another 300 gm. The administration of carbonate was continued in doses varying from 2 to 5 gm. until a total of 51.18 gm. of glucose had been retained. 2 hours before the animal was anesthetized 50 gm. of glucose and 5 gm. of sodium carbonate were given by stomach tube, thus supplying a large quantity of glucose for glycogenesis and of sodium carbonate to inhibit any glycogenolysis that might be caused by the etherization in the normal animal (8). Care was taken to allow sufficient air during the etherization and the excitement stage was thereby reduced. 9 minutes elapsed from the time that the anesthetic was begun until the first specimen of liver was removed and placed in the alkali solution. Glycogen was determined by the method of Pflüger. After prolonged hydrolysis with dilute hydrochloric acid the fluid was concentrated to a small volume by slow evaporation on the water bath, neutralized after cooling, and the sugar content determined by Benedict's titration method. As shown in the protocol of this experiment at the bottom of Table I, only amounts of sugar too small for quantitative estimation by Benedict's method could be demonstrated in the fluid after hydrolysis.

Experiment 2 (*Table II*).—This is reported in corroboration of the above except that in the second experiment no anesthetic was used in the final procedure.

TABLE I.

Experiment 1. Dog 6. Female. Weight 13.87 Kg. Depancreatized in Two Stages: First Stage, November 22, 1914; Second Stage, Transplant Removed December 4, 1914.

Date.	Time.	Total glucose.		Total nitrogen.	Glucose per hr.	Nitrogen per hr.	G : N	Reaction.	Temperature.	Remarks.
		Titration.	Polariscope.							
1914		*gm.*	*gm.*	*gm.*					*°C.*	
Dec. 6	3.30 p.m. to									Water.
" 7	3.10 p.m.	32.40	34.49	11.872	1.467	0.503	2.90	Alkaline.	38.4	
" 10	9.30 a.m. to									2 gm. anhydrous sodium carbonate in 200 cc. H_2O at 10 a.m. and
	7.30 p.m.	9.39	9.52	3.58	0.939	0.358	2.62	"	38.5	1 p.m. by stomach tube.
" 10	7.30 p.m. to									Wound healing, clean. 2 gm. Na_2CO_3 in 200 cc. H_2O by stom-
" 11	7.30 p.m.	15.30	15.71	8.03	0.637	0.334	1.90	"	38.4	ach tube 7.40 p.m. Dec. 11. Retained 6.86 gm. glucose.
	7.30 p.m. to									At 10 a.m. 2 gm. Na_2CO_3 in 300 cc. water and at 2.10 p.m. by
" 12	10 a.m.	11.53	12.85	5.51	0.623	0.297	2.09	"		stomach tube. Retained 3.740 gm. glucose.
	7.30 p.m.	Urine contaminated with feces; discarded. Catheterized 7.30 p.m.								300 gm. lean beef heart given; all taken 7.40 p.m.; also 2 gm. Kahlbaum Na_2CO_3 in 300 cc. water. Feces analyzed for sugar; negative.
	7.30 p.m. to									400 gm. lean beef heart at 7.30 p.m. + 4 gm. Kahlbaum Na_2CO_3.
" 13	7.20 p.m.	30.34		17.86	1.264	0.744	1.69	Alkaline.	38.2	Retained 18.96 gm. glucose.

Dec. 14	7.20 p.m. to 7.15 p.m.	32.14		16.04	1.339	0.668	2.00	Alkaline.		Feces analyzed for sugar; negative. 5 gm. Kahlbaum anhydrous Na_2CO_3 and 300 gm. lean beef heart at 7.30. Retained 12.14 gm. glucose.
" 15	1.20 p.m.	16.87		9.54	0.937	0.530	1.76	"	38.4	At 1.40 p.m. 50 gm. Merck's c.p. dextrose + 5 gm. Kahlbaum sodium carbonate (anhydrous) given by stomach tube. All taken and none vomited. Retained 9.48 gm. Dog weighed 9.5 kg.

Dec. 15. Anesthetic (ether) started at 3.43 p.m. No excitement stage; allowed sufficient air. 3.52 p.m. First specimen of liver removed and in alkali solution 75 gm. 3.55 p.m. Second specimen of liver removed and in alkali solution 60 gm. 4.07 p.m. Third specimen of muscle removed and in alkali solution 75 gm. Dog stopped breathing 3.54 p.m. Specimens all contained but a faint trace of sugar after hydrolysis. Total glucose retained 51.18 gm. One urine period lost (contamination). 2.76 used as control G: N.

TABLE II.

Experiment 2. Dog 10. Female Terrier. Weight 4.6 Kg. Depancreatized in Two Stages: First Stage, May 2, 1916; Second Stage, Transplant Removed May 8, 1916.

Date.	Time.	Glucose.	Nitrogen.	G : N	Blood sugar.	Weight.	Reaction.	Remarks.
1916		*gm.*	*gm.*		*per cent*	*kg.*		
May 8–9	3 p.m.–10 a.m.	13.09	3.14	4.17	0.245	4.6	Acid.	150 gm. lean meat.
" 9–10	10 a.m.–10 a.m.	25.25	8.01	3.15	0.214	4.4	"	
" 10	1 p.m.							35 gm. glucose in 200 cc. water. Some lost.
	5 "							180 cc. of 1 per cent Na_2CO_3 in Ringer's solution intravenously.
	5.30 p.m.							150 gm. lean meat.
" 11	10 a.m.	25.23	3.75	6.72	0.346		Alkaline.	Dog bled from wound at 8 a.m. Some urine lost.
	2–2.15 p.m.							150 cc. of 1 per cent Na_2CO_3 in Ringer's solution intravenously.
" 12	10 a.m.	42.18	3.95	10.7	0.301	4.0	"	44 gm. glucose *per os*. 14.76 gm. glucose retained.
	10.45–11 a.m.							Injected 200 cc. of 1 per cent Na_2CO_3 in Ringer's solution intravenously.
	11.30 a.m.							44 gm. glucose *per os*.
	6 p.m.							Injected 150 cc. of 1 per cent Na_2CO_3 in Ringer's solution intravenously.
	6.35 p.m.							Dog killed suddenly.

Temperature was normal during the entire experiment, 38–39°.

Results of analyses for glycogen, May 12:

6.35 p.m. Specimen 1 (liver) ground and placed in 60 per cent KOH = 0.092 per cent.
6.36 " " 2 " " " " " 60 " " = 0.059 "
6.40 " " 3 (muscle) " " " " 60 " " = None.
6.43 " " 4 (heart muscle) ground and placed in 60 per cent KOH = 0.675 per cent.

Glycogen was determined by Pflüger's method. The solution was made acid up to 2.2 per cent with HCl, and heated for 3 hrs. with a reflux condenser over the boiling water bath. Sugar was determined by the method of Lewis and Benedict.

Experiment 3 (*Table III*).—The animal was completely depancreatized in two stages and the urine collected in definite periods of approximately 24 hours' duration, the urine, as seen in Table III, showing a G: N of 3.07 for the first 23 hours and 2.98 for the next 24 hours. Adrenalin in 1: 1,000 dilution was then administered subcutaneously every 3 hours in doses of 0.25 to 0.5 cc. until further administration of adrenalin failed to raise the G: N. This was taken as indicating that the animal was now completely deglycogenized (7). The adrenalin was then discontinued and sodium carbonate in 1 per cent solution was given by stomach tube in repeated doses of 2.5 to 5 gm.

The dog was fed on lean beef in quantities varying from 200 to 500 gm. daily in one or two feedings. Even before the meat feeding was begun the G: N dropped from 2.66 to 2.31 following the administration of sodium carbonate. The G: N continued to fall even during the period in which the meat was being fed. Thus a considerable amount of glucose that must have been derived from the ingested meat failed to appear in the urine.

The adrenalin injections were then resumed with the object of bringing about the elimination of the supposedly retained glucose, as extra sugar, which would have been the case if it were retained as glycogen. That no such extra glucose appeared even after the injection of 0.5 cc. of adrenalin at 3 hour intervals, we are inclined to interpret as conclusive proof that glucose retained under the influence of sodium carbonate does not exist as glycogen. For suggesting this method of procedure we are indebted to Dr. Woodyatt.

The negative result in Experiment 1 and in other experiments similarly performed led us to think that the sugar which was apparently retained might have been excreted through other channels. Therefore, the possibility of glucose being excreted by the saliva, stomach, and intestine was investigated.

hrs. with a reflux condenser over the boiling water bath. Sugar was determined by the method of Lewis and Benedict.

TABLE III.

Experiment 3. Dog 8. Female Bull Pup. Weight 8.4 Kg. Pancreatectomy: First Stage, February 26, 1915; Second Stage, March 3, 1915.

Date.	Time.	Glucose.		Nitrogen.	G : N	Weight.	Acetone.		Remarks.
		Titration.	Polariscope.				Gunning.	Rothera.	
1915		*gm.*	*gm.*	*gm.*		*kg.*			
Mar. 3–4	2.15–1.15 p.m.	32.02	32.12	10.42	3.07*	8.4	+	−	
" 4–5	1.15–1.30 "	34.89	32.12	11.70	2.98*		+	++	Adrenalin. See note 1 below.
" 5–6	1.30–1.30 "	33.88	32.12	10.47	3.23*	8.4	+	++	
" 6–7	1.30–1.20 "	33.79	31.39	11.73	2.88*	8.76	+	++++	
" 7–8	1.20–1.35 "	31.03	30.65	11.63	2.66*	7.75	+	++	
" 8	2.00 "								250 cc. of 2 per cent Na_2CO_3 by tube; partly vomited.
	5.30 "								500 cc. of 1 per cent Na_2CO_3 by tube.
	6.00 "	2.51	2.57	1.11	2.31**				
" 9	1.35 "	26.66	26.60	11.09	2.40**	8.00	−	−	See note 2.
" 9–10	1.35–1.35 "	26.26	26.16	13.86	1.89**	7.59	−	−	Lean meat and carbonate.
" 10–11	1.35–1.35 "	29.61		12.85	2.30**	7.37	−	−	
" 11–12	1.35–1.25 "	19.64	20.24	7.64	2.57**	6.69	+	+	Adrenalin.
" 12–13	1.25–1.30 "	21.97	18.33	7.62	2.88*	6.35	−	−	See note 3.
" 13	5.00 "	31.59		15.12	2.08**		−	−	Fed 600 gm. lean meat and 6 gm. sugar-free extract of pancreas.

* Urine acid.

** Urine alkaline.

1. 0.3 cc. adrenalin injected subcutaneously every 3 hrs. from Mar. 5, 1915, at 4.30 p.m. to Mar. 8 at 10.30 a.m.

2. Administered 25 gm. of sodium carbonate in 2.5 gm. doses, and 1,050 gm. lean meat *per os* in divided amounts from Mar. 8 at 9 p.m. to Mar. 11 at 1.40 p.m.

3. Adrenalin injections resumed on Mar. 11 and repeated every 3 hrs. until Mar. 13 at 10 a.m.

4. Dog anesthetized at 1.50 p.m. on Mar. 14. Right Wharton's duct exposed, cannula inserted, and about 2 cc. of saliva obtained which was found to contain sugar by both the Benedict and Fehling tests.

5. At least 21.51 gm. of glucose retained under the influence of sodium carbonate.

Appendix to Table III.

Mar. 14. Final experiment on Dog 8.

1.35–1.47 p.m. Saliva collected (ether stimulation); free flow. Safranin test positive, Benedict test negative. Fehling's positive after standing over night.

1.50 p.m. Ether anesthesia begun. Trachea exposed, tracheal cannula inserted and tied in.

1.58–2.02 p.m. Left femoral artery and vein exposed and cannulas tied in.

2.03–2.15 p.m. Abdomen opened. Liver found shrunken but of normal color and consistency and adherent to duodenum. Abdominal cavity clean but intestines matted together in adhesions at site of removal of pancreas. No pancreatic remnants found. Adhesions broken up, tube inserted into stomach and tied into esophagus, cannulas tied into pyloric ends of stomach and duodenum and lower end of ileum.

2.30 p.m. Stomach and intestine washed with normal saline and washings discarded.

2.45 p.m. Duct of right submaxillary gland exposed, cannula inserted. Chorda tympani nerve exposed and stimulated electrically (indirect interrupted current) 3.15–3.35 p.m., and saliva collected. A few drops of this saliva (mucin-free) gave a voluminous precipitate both with Benedict's and Fehling's reagents.

3.25 p.m. Both stomach and intestine again irrigated, the former with 500 cc., the latter with 1,000 cc. of 0.9 per cent NaCl. Washings collected, acidified with acetic acid, heated to remove protein, concentrated to less than 100 cc., neutralized and rendered alkaline with sodium carbonate, and tested for sugar. None found.

3.21 p.m. Blood collected from left femoral artery for determination of specific gravity and blood sugar.

Specific gravity.... {1.060 Hammerschlag method, using
1.058 chloroform and benzene.

Sugar analysis. .....0.327 per cent.

3.30 p.m. Injection of 1 per cent Na_2CO_3 solution into left femoral vein begun. Rate 3 to 8 cc. Total amount injected 200 cc.

3.40 p.m. Catheterized, about 50 cc. of acid urine obtained.

5.25 p.m. Injection of carbonate finished.

5.20 p.m. Chorda tympani nerve again stimulated and second sample of saliva collected, which also gave both strong Benedict and Fehling tests. Not as strong as the first with the same amount of saliva and reagent.

5.26 p.m. Blood collected for specific gravity and blood sugar.

Specific gravity..... {1.047
1.050

Blood sugar.....0.378 per cent.

5.30 p.m. Stomach and intestine washed with normal salt solution as before. No sugar found either by Benedict's or Fehling's test.

5.40 p.m. Bladder catheterized, no urine obtained. At autopsy the bladder was found contracted and empty.

5.48 p.m. Dog killed.

Examination of Saliva.

The saliva of Dog 8 (Experiment 3), as well as that of the dog used in Experiment 5, was collected at least once each day, the quantity obtained varying from 5 to 20 cc. When the saliva did not flow spontaneously, a flow was brought about by ether fumes blown into the mouth. Mucin was removed by adding acetic acid to the mixed saliva and the filtrate tested by at least one of three tests, Benedict, Fehling, or safranin, or by all three.

At the end of Experiment 3 the animal was anesthetized, the duct of the right submaxillary gland exposed, a cannula inserted into it, and saliva obtained by stimulating the chorda tympani nerve electrically at definite intervals, as indicated in Table III.

In Experiment 5 the pancreas was removed in two stages at an interval of 4 days, and when the G: N ratio reached the level of 2.89 saliva was collected and tested for sugar, with negative results. Further work on this dog was rendered impossible by the development of complications. It will be noted that even when the elimination of glucose in the urine was maximal the saliva contained no sugar (Table IV), nor were we able to demonstrate any appreciable amount of sugar after feeding meat and carbonate.

Examination of Feces and Intestinal Secretions.

Two specimens of feces were collected during the latter part of the experiment reported in Table I. These were extracted with acidulated water; the acid extract, heated to coagulate the protein, was then filtered, and the filtrate concentrated to small volume; it was then cooled, neutralized, and examined for sugar, but none was found in either instance.

In Experiment 3, as shown in the protocol, the abdomen was opened, cannulas were tied into the pyloric end of the stomach and duodenum and also into the lower end of the ileum. The stomach and intestine were washed at definite intervals with 1 liter of warm salt solution both before and after the intravenous injection of a 1 per cent sodium carbonate solution. The blood was also collected before and after the administration of the carbonate, and the blood sugar and specific gravity were determined. The changes observed in the blood are due to failure of the kidney.

All the washings were acidified with acetic acid, heated to coagulate the protein, filtered, the filtrate was concentrated to 100 cc. or less, and then made up to 100 cc., and the sugar content determined by Benedict's method. Results were negative. (See appendix to Table III.)

TABLE IV.

Experiment 5. Dog 10. Female Bull Terrier. Pancreatectomy: First Stage, April 29, 1915; Second Stage, May 3, 1915.

Date.	Time.	Total glucose.		Total nitrogen.	Glucose per 24 hrs.	Nitrogen per 24 hrs.	G : N	Temperature.	Saliva.	Remarks.
		Titration.	Polariscope.							
1915		*gm.*	*gm.*	*gm.*				*°C.*		
May 3–4	1.45 p.m.									Water.
	4.30 "	34.37	35.09	11.87	30.72	10.63	2.89	38.2		
									Safranin and Benedict tests negative.	
" 4–5	2.30 "	32.37	32.12	11.34	35.32	12.36	3.03			

TABLE V.

Experiment 6. Dog 7. Female, Weight 9 Kg. Depancreatized, February 6, 1915.

Date.	Time.	Total glucose.		Total nitrogen.	G : N	Temperature.	Remarks.
		Titration.	Polariscope.				
1915		*gm.*	*gm.*	*gm.*		*°C.*	
Feb. 8	A.m. specimen.	15.87	15.76	5.18	3.04	39.5	300 gm. lean beef heart.
" 9	" urine.	37.20	35.71	12.54	2.96		Fed 200 gm. beef heart and 3 gm. extract of pancreas.
" 10							Urine lost. 0.25 cc. 1: 1,000 adrenalin (Parke, Davis and Co.) subcutaneously for four doses.
	8 p.m.	21.64	20.24	4.53	4.77	40.5	
" 11	A.m. specimen.		2.08	1.23	1.69		
	1.15 p.m.		2.08	2.55	0.81	39.0	
" 12	1.15 "		5.95	5.01	1.18	39.8	4 p.m., fed 300 gm. lean meat. Vomited 150 gm.
" 13	2.00 "	17.26		6.19	2.78		

Appendix to Table V. Experiment 6.

2.10 p.m. Dog anesthetized. Left Wharton's duct exposed and cannula inserted. Chorda tympani nerve of same side also exposed.

2.40 p.m. Chorda tympani stimulated for 10 minutes and saliva collected. Contained trace of glucose (Benedict's test).

2.50 p.m. Cannulas tied into pyloric end of stomach and duodenum and lower end of ileum; also stomach tube inserted into stomach.

3.50–4.00 p.m. Stomach washed with 1,000 cc. of 0.9 per cent sodium chloride solution; 900 cc. recovered. No reduction found after removing proteins by acid coagulation and heat and concentrating to 100 cc.

4.00–4.10 p.m. Small intestine washed. About 900 cc. of fluid recovered. No reducing substance found after similar treatment.

4.15–4.50 p.m. 160 cc. of 16.36 per cent solution of c. p. glucose (Merck's anhydrous) injected into left femoral vein.

4.55–5.05 p.m. Second intestinal irrigation. After similar treatment as first stomach washing, the fluid reduced Benedict's solution. Amount too small for quantitative estimation by Benedict's method.

5.05–5.17 p.m. Second stomach washing—1,000 cc. of solution used. Only 300 cc. recovered. No reducing substances present.

5.20–5.45 p.m. 170 cc. of 1 per cent anhydrous sodium carbonate injected into left femoral vein.

5.40–5.50 p.m. Third stomach washing. No reducing substances detected in wash. All fluid recovered.

5.50–6.00 p.m. Third intestinal washing contained a quantity of reducing substance equal to 0.104 gm. of glucose. All fluid recovered.

5.15 p.m. Chorda tympani stimulated and saliva collected. Gave strongly positive test with Benedict's qualitative solution. Two drops of saliva gave positive test.

Autopsy.—No peritonitis. Sloughing at site of adrenalin injection. Lower part of ileum adherent to abdominal wound.

The first part of Experiment 6 is reported as a control showing that when lean meat is fed to a dog after repeated injections of adrenalin the G: N is approximately 3.00. Pancreatectomy was performed on Feb. 6, 1915. On Feb. 8, analysis of a specimen of urine obtained in the morning yielded a G: N of 3.04. The dog was fed 300 gm. of lean beef heart and 3 gm. of extract of pancreas which was sugar-free. 200 gm. of beef heart and 1 gm. of pancreatic extract were fed after removing the morning urine. The urine on analysis gave a G: N of 2.96. The urine of the 10th was lost. Four doses of adrenalin were injected subcutaneously at 3 hour intervals on the 10th, and the urine obtained at 8 p.m. on the same day showed a G: N of 4.77. Adrenalin injections were then discontinued and on the 11th and 12th three specimens of urine gave G: N ratios varying from 0.81 to 1.69. But after the administration of 300 gm. of lean meat, of which 150 gm. were vomited, the ratio again returned to 2.78, the typical Minkowski ratio.

The procedure in the second half of this experiment was similar to that adopted in Experiment 3. The stomach and intestinal contents were washed out before and after infusion of glucose into the vein, and again after infusion of 1 per cent sodium carbonate. Only in one instance was any reducing substance obtained, and this may have been due to failure of the kidney as in Experiment 3.

In Experiment 7 the procedure was similar to that in the third experiment and here again, although 25.58 gm. of glucose were retained following carbonate injections, none of it appeared in the urine as extra glucose during the second course of adrenalin injections.

TABLE VI.

Experiment 7. Dog 21. Depancreatized in Two Stages: 29.9 Gm. of Pancreas Removed February 29, 1916. Remaining 6.8 Gm. Removed March 3, 1916. Weight 14.16 Kg.

Date.	Time.	Total glucose.		Nitrogen.	G : N	Blood sugar, volume per cent.	Weight.	Reaction.	Remarks.
		Titration.	Polariscope.						
1916		*gm.*	*gm.*	*gm.*			*kg.*		
Mar. 14–15	10 a.m.–10 a.m.	27.565	27.50	8.97	3.07	0.340		Acid.	
" 16	10 a.m.	20.045	20.62	6.89	2.90	0.346	12.36	"	0.3 cc. adrenalin injected subcutaneously every 3 hrs. from 11 a.m., Mar. 16–2 p.m., Mar. 18.
" 17	10 a.m.	45.454	42.98	8.96	5.07	0.336	12.24	"	
" 18	4 p.m.	25.67	25.90	9.70	2.64	0.336		"	Dog very weak. Expelled black feces (blood).
	4.15–4.35 p.m.								250 cc. 1 per cent Na_2CO_3 in Ringer's solution intravenously. Distinct improvement in general condition at once.
	4.50 p.m.								50 gm. glucose offered. All taken at once.
" 19	4.00 "	60.09	57.56	10.20	5.88	0.377			13.15 gm. glucose retained.
	4.20–4.45 p.m.								350 cc. 1 per cent Na_2CO_3 in Ringer's solution intravenously followed by 40 gm. glucose *per os*.
" 20	4.10 p.m. 5.00 "	47.21	47.74	7.014	6.73	0.241	11.50	Alkaline.	12.43 gm. glucose retained. 0.3 cc. adrenalin injected subcutaneously every 2 hrs.
" 21	4.10 "	18.23	17.16	6.85	2.66			Acid.	Total amount of glucose retained 25.58 gm. Temperature fluctuated between 37.9 and 39.5°; at 4.10 p.m. on Mar. 21, was 35°.

40 4:10 p.m. on Mar. 21, was 35°.

DISCUSSION.

Sansum and Woodyatt (7) observed the constant increase of the G:N varying from 3.7 to 6 in completely phlorhizinized dogs on administering ether, nitrous oxide, or other narcotics. Thus, in one experiment etherization for 10 minutes yielded 3.88 gm. of extra glucose. In a second experiment, 30 minutes of anesthesia brought about an elimination of 7.98 gm. of extra sugar; and in the third experiment the same dog eliminated 11.26 gm. of extra sugar during a 2 hour anesthesia. This extra sugar they showed to be derived from preformed glycogen. Hence, it may be contended that etherization favors the mobilization of glycogen, for Macleod and Pearce (9) have shown that in anesthetized dogs there is a rapid disappearance of glycogen from the liver. Nevertheless, it is difficult to imagine, in view of Sansum and Woodyatt's results, that more than a small fraction of the 51.18 gm. of retained glucose in our Experiment 1 could have been mob lized in the short period of 9 minutes which elapsed from the time the anesthesia was begun until the first specimen of liver was removed. It might also be contended that the sugar was retained as glycogen, deposited to a large extent in the heart rather than in the liver or in the muscle. Cruickshank (10) has shown that, while the percentage of glycogen in hearts of normal dogs may be as high as 0.5 per cent, in depancreatized dogs it may be increased up to 1 per cent. That these assumptions will not suffice to explain the results obtained by the present writers is evident from Experiment 3 in which the second series of injections of adrenalin failed to bring about an excretion of extra sugar. The glycogen content of the heart muscle, as reported in Experiment 2, agrees well with similar analyses reported in Cruickshank's paper.

Carlson and Ryan (11) have reported the finding of glucose in the saliva of normal cats, the quantity of sugar present depending upon the gland from which the saliva was obtained. The quantity was considerably increased by the administration of ether but was present in appreciable amounts even in docile, unanesthetized cats, so that sugar could be readily detected by the ordinary Fehling test. These writers also report the case of a man whose urine contained more than 3.5 per cent of sugar. His saliva gave a positive safranin test although phenylhydrazine and Fehling's tests were both negative. In the animals experimented on there seemed to be a proportionality between the concentration of the sugar in the blood and the amount of glucose in the saliva. In 1904 MacCallum (12) reported the observation that the intravenous infusion of a M/6 solution of sodium chloride in sufficient quantity produces a hyperglycemia in rabbits. When the blood sugar reaches a certain concentration glucose is excreted by the kidneys, and if the kidneys are cut off from the circulation the glucose is excreted by the intestine and stomach. In a subsequent experiment MacCallum found sugar being excreted into the intestinal fluid even though the kidneys were actively functionating. He speaks of an "intestinal diabetes" and a vicarious functioning of the intestine in the elimination of glucose when the renal function is defective.

Fisher and Moore (13) were unable to confirm MacCallum's observations. They showed that simple hyperglycemia, induced by the hypodermic injection of morphine, by diabetic puncture, or by the intravenous injection of glucose or sucrose, never leads to an elimination of sugar into the gastro-intestinal tract. But if a solution of sodium chloride is injected into the animals thus treated in quantity and concentration which of itself does not bring about the excretion of sugar into the intestinal tract, sugar appears in detectable quantities in the stomach and intestine. They therefore explain MacCallum's results as being due to the combined effect of morphine and sodium chloride and interpret their own results as being attributable to the influence of the sodium ion in increasing the permeability of the intestinal epithelium.

The most recent work on the excretion of dextrose into the stomach and small intestine is that of Kleiner (14) who found definitely measurable quantities of dextrose in the small intestines of normal rabbits, but the experimental procedure adopted by Kleiner, which involved anesthesia, operation, and asphyxia by clamping the trachea, would tend to produce a high degree of hyperglycemia. Kleiner found more sugar in the intestine after injecting as much as 7 gm. of glucose per kilo of body weight in 20 per cent solutions than when 4 gm. of glucose per kilo in 11.4 per cent solution were injected. Furthermore, while 0.5 per cent of injected dextrose appeared in the intestines when the kidneys were active and the larger quantity of glucose was given, 2.2 per cent appeared when the smaller amount was administered and the kidneys cut out of the circulation.

Both Experiments 3 and 6, of our own series, seem to show clearly that in depancreatized dogs but little reducing substance is excreted into the small intestine and none into the stomach. And while the amount eliminated by way of the small intestine may be somewhat increased by the injection of large amounts of glucose into the circulation, it is nevertheless so small as to exclude any vicarious functioning of the gastro-intestinal tract in the excretion of sugar, even when the glycemia is very high. Likewise, the quantities of glucose eliminated by our diabetic dogs by way of the saliva were so small, even when the hyperglycemia must have been very high as a result of the combined effects of removal of the pancreas and injections of adrenalin, as to have no bearing upon our problem. On the other hand, when the amount of sugar in the blood is still further increased as a result of anesthesia or the direct introduction of glucose into the circulation in massive doses, and particularly when renal function is depressed by the anesthetic or exposure, then considerable amounts of glucose may be excreted by the saliva.

It would seem that the anesthetic (ether) increases the permeability of the epithelium of the salivary glands to the sugar of the blood.

While it is realized that the number of experiments reported does not justify sweeping conclusions, nevertheless the following may be tentatively suggested. (1) Glucose retained as a result of the administration of sodium carbonate to depancreatized dogs is not held back as glycogen; and (2) the sugar is not excreted by way of the saliva, stomach, or small intestine. The question whether it is oxidized and other related questions will be treated in the later papers of this series.

In conclusion we wish to thank Professor J. T. McClintock for his cooperation in this research and also others who kindly gave us their assistance in the operative work.

BIBLIOGRAPHY.

1. Pavy, F. W., and Bywaters, H. W., *J. Physiol.*, 1910, xli, 168.
2. Ehrlich, P., *Arch. ges. Physiol.*, 1908, cxxi, 236.
3. Elias, H., *Biochem. Z.*, 1912–13, xlviii, 120.
4. Elias, H., and Kolb, L., *Biochem. Z.*, 1913, lii, 331.
5. Macleod, J. J. R., and Pearce, R. G., *Am. J. Physiol.*, 1909, xxiii, 278.
6. Murlin, J. R., and Kramer, B., *J. Biol. Chem.*, 1916, xxvii, 481; also 1913, xv, 365.
7. Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.*, 1915, xxi, 1.
8. Pavy, F. W., and Godden, W., *J. Physiol.*, 1911, xliii, p. vii.
9. Macleod and Pearce, *Am. J. Physiol.*, 1910–11, xxvii, 341.
10. Cruickshank, E. W. H., *J. Physiol.*, 1913–14, xlvii, 1.
11. Carlson, A. J., and Ryan, J. G., *Am. J. Physiol.*, 1908, xxi, 301.
12. MacCallum, J. B., *Univ. California Publications, Physiology*, 1904, i, 125.
13. Fisher, M. H., and Moore, G., *Am. J. Physiol.*, 1907, xix, 314.
14. Kleiner, I. S., *J. Exp. Med.*, 1911, xiv, 274.

PANCREATIC DIABETES IN THE DOG.

III. THE INFLUENCE OF ALKALI ON THE RESPIRATORY METABOLISM AFTER TOTAL AND PARTIAL PANCREATECTOMY.

By J. R. MURLIN and B. KRAMER.

With the technical assistance of J. A. Riche.

(*From the Physiological Laboratory, Cornell University Medical College, New York City.*)

(Received for publication, September 29, 1916.)

The preceding papers have shown that alkali may cause a complete disappearance of sugar from the urine of an animal after pancreatectomy or may cause the retention of a considerable amount of ingested glucose without, however, leading to the formation of glycogen. The natural inference is either that oxidation of the sugar is favored or the inflow of sugar from the tissues to the circulation is retarded to a rate slower than that by which it makes its escape from the body by the kidneys. Either effect would be beneficial to an organism suffering from a diminished pancreatic function, and the subject merited careful study.

The investigation of the respiratory metabolism after administration of sodium carbonate, the salt which has given best results in the study of the glycosuria and hyperglycemia, is attended with peculiar difficulties. The sign of improved oxidation of glucose is a rise in the respiratory quotient. But if any carbon dioxide were to be given off by the salt itself, whether set free in the stomach by hydrochloric acid and vomited, or set free by neutralization in the blood with acid phosphate and eliminated through the breath, a rise in respiratory quotient of no moment for the real processes of metabolism would be obtained. The most careful controls had consequently to be made and the experimental work has consumed an immense amount of time.

Our conclusion after some 3 years' intermittent occupation with the problem is that in the totally depancreatized dog in good condition, *i.e.*, not moribund, the alkali alone is almost

always without effect on the respiratory metabolism. An increase of a few points at most in the respiratory quotient may be obtained. With the partially operated animal, however, the result is striking and immediate. Experiments in sufficient number to illustrate these facts will be presented in detail. In the interest of brevity many negative results will be omitted entirely.

We have obtained some evidence, also, that pancreatic material (whether merely as an extract or in virtue of the presence of living cells we are not yet prepared to say) is, when administered with alkali, of distinct benefit to the diabetic organism. Because of the impossibility of longer continuing our work jointly we are obliged to leave this and some other points at an inconclusive stage.

The significant experiments on animals anatomically wholly depancreatized may be classified as follows: (A) negative effects; (B) experiments of doubtful import; (C) positively favorable effects. On partially depancreatized animals we have only two experiments but they are both positive; that is to say, both show conclusive evidence of improved oxidation of glucose. Believing that these results are of hopeful augury in the treatment of diabetes, we present them in full.

Criteria of Improved Oxidation.

Throughout this work we have relied not merely upon the respiratory quotient but, wherever possible, have calculated the heat production as well and when the after effect could be compared with a basal metabolism have taken note of the dynamic action of the ingested glucose (1).

The criterion of the completely diabetic state from pancreatectomy is a respiratory quotient of about 0.69. Lusk (2) has shown that the G:N ratio which Minkowski found to be typical of the depancreatized dog on a meat diet, namely, 2.8, should correspond to a respiratory quotient of 0.697. La Franca (3), Falta, Grote, and Staehelin (4), and the writers (5) have elsewhere reported quotients for totally depancreatized dogs varying from 0.67 to 0.71. Many quotients confirmatory of these observations will be found in the following pages. A discussion of the

relationship between respiratory quotient and G: N ratio is reserved for a later paper. The experimental procedure has usually been the following. After establishment of the true diabetic respiratory quotient (and basal metabolism), alkali alone or alkali with glucose was administered, sometimes by stomach tube, sometimes by intravenous infusion, and the animal was returned to the respiration machine for 1 to 4 hours' observation. In a few instances, not here recorded, the time has been extended to 6 hours.

Experiments on Totally Depancreatized Dogs.

A. Negative Effects.

Dog 3, a young female bull terrier, weighing about 8 kg., was operated on July 18, 1914. 3 days later the G: N ratio on a meat diet was 2.79. The same day R. Q.'s of 0.71 were obtained before and after administration of 17.89 gm. of pure dextrose by stomach.[1] On the 22nd, 5 gm. of sodium carbonate in 250 cc. of water were given by stomach, and quotients of 0.70, 0.71, and 0.69, successively, were obtained in the 4 hours immediately following. On the 23rd, with the G: N ratio at 1.89, the following experiment was carried out.

TABLE I.

Dog 3. Weight 8 Kg. Pancreatectomy, July 18, 1914.

Date.	Weight.	Time.	Respiratory metabolism.			Remarks.
			CO_2	O_2	R. Q.	
1914	*kg.*	*p. m.*	*liters*	*liters*		
July 23	7.7	3.42–4.27	2.53	3.66	0.69	2.10–2.30 1.5 gm. Na_2CO_3 (anhydrous) in 200 cc. distilled water infused by vein.
		4.27–5.13	2.68	3.97	0.68	
		7.18–8.08	2.76	3.82	0.72	6.12–6.35 1.8 gm. Na_2CO_3 (anhydrous) in 100 cc. distilled water into femoral vein; 10 gm. glucose by stomach tube.
		8.03–8.49	2.85	4.17	0.68	

It is possible, judging from subsequent experience, that not enough glucose was given in this instance to insure penetration of the intestinal wall in sufficient amount to produce a pronounced

[1] See Dog 3, Paper I of this series.

rise in R. Q. The slight increase from 0.68 to 0.72, beginning within an hour after the infusion ceased, is not regarded as of great significance.

Dog 54, a female fox terrier, was depancreatized by the two stage operation. All the organ except a small portion of the uncinate process engrafted under the skin at the abdominal incision was removed on June 23, 1916. For a week the dog was fed the mixed diet employed by one of us in a series of papers on the pregnancy of the dog (6) and known in the papers of Professor Lusk as "the standard diet." On June 30 the graft was removed and the animal promptly became diabetic. Some preliminary observations on July 3 and 5 proved that the dog still had some capacity to oxidize glucose but on the 6th this capacity, as judged by the respiratory quotient, had wholly disappeared.

TABLE II.

Dog 54. Female Fox Terrier. Weight 7 kg. Pancreatectomy: First Stage, Graft under Skin, June 23, 1916; Second Stage, June 30, 1916. Mixed Diet between Operations.

Date.	Weight.	Temperature.	Time.	Respiratory metabolism.			Remarks.
				CO_2	O_2	R. Q	
1916	*kg.*	*°C.*		*liters*	*liters*		
July 6	5.7	38.2	10.46–11.46	2.07	2.90	0.71	Basal, *i.e.*, following food evening before.
			11.46–12.46	2.34	3.44	0.68	1 p.m. 30 gm. glucose in 200 cc. Ringer's solution + 0.05 N NaOH by stomach.
			1.49– 2.49	2.53	3.62	0.70	
			2.49– 4.05	3.71	5.11	0.71	
			4.05– 5.05	2.79	3.98	0.70	Dog urinated or vomited 2.05, after 2.50, and during third period.
" 8	5.75	38.0	10.17–11.24	2.34	3.20	0.71	Basal.
			11.24–12.19	2.22	3.03	0.71	12.40, 30 gm. glucose in 200 cc. Ringer's solution + 0.05 N NaOH by stomach.
			3.01– 4.00	2.57	3.71	0.69	
			4.00– 5.00	3.04	4.32	0.70	
			5.00– 5.58	2.94	4.13	0.71	Urinated during every period, but lapped it up at once.

TABLE II—*Concluded.*

Date.	Weight.	Temperature.	Time.	Respiratory metabolism.			Remarks.
				CO_2	O_2	R. Q.	
1916	*kg.*	*°C.*		*liters*	*liters*		
July 9							2.15 p.m. 200 cc. Ringer's solution + 0.05 N NaOH + pancreas extract by stomach.
" 10	5.8	38.4	10.47–12.04	2.66	3.91	0.68	Basal.
			12.04– 1.04	2.31	3.22	0.72	
							2.30 p.m. 200 cc. pancreas extract in Ringer's solution + 0.05 N NaOH by stomach tube.
			2.45– 4.00	2.66	3.79	0.70	
			4.00– 4.40	1.78	2.55	0.70	
" 11	5.9	38.2	11.45–12.15				150 cc. pancreas extract in Ringer's solution + 0.05 N NaOH intravenously.
							12.35 p.m. 30 gm. glucose by stomach tube.
			12.52– 1.52	2.62	3.74	0.70	
			1.52– 2.52	3.57	5.01	0.71	

We shall see later that a similar treatment with pancreatic substance in Ringer's solution, made alkaline to 0.05 N with pure sodium hydroxide, given with sugar gives in some instances a marked rise of quotient and of heat production. In the present instance, however, it failed completely. So far as is known, the experimental conditions were good except for the fact that the animal urinated frequently in the metabolism cage. The dog was always quiet at the termination of the periods and we have always found that the residual analyses for water and carbon dioxide in the air of the cage, with corrections for temperature and pressure variations which we employ, are reliable (7). Our only explanation for complete failure in this instance is that

the latent time[2] within which the favorable effect of alkali may be expected varies considerably in different animals, and that the present case is one in which a much longer time than we allowed might be necessary.

B. Experiments of Doubtful Import.

Two of our experiments on the respiratory metabolism, while positive in character, are of doubtful import because of peculiar circumstances.

Dog 7 (Paper I, page 489), a black female, weighing about 4 kg., had refused all food for 4 days previous to operation and had continued to fast for 2 days, whereupon the urine was found to be free of sugar. On the 2nd day sodium bicarbonate had called out in 2 hours sugar in sufficient amount to make a G: N ratio of 2.61 (page 489). On the 8th day following pancreatectomy the dog had excreted in 24 hours only 13.68 gm. of glucose out of 17.89 gm. of pure dextrose (Merck) given by stomach tube and had yielded respiratory quotients indicating combustion of some carbohydrate. On the 28th, the 9th day, the experiment below was carried out.

TABLE III.

Dog 7. Weight 4 Kg. Pancreatectomy, August 19, 1914.

Date.	Time.	Respiratory metabolism.			Remarks.
		CO_2	O_2	R. Q.	
1914		*liters*	*liters*		
Aug. 28	10.30 a.m.				Given 20 gm. glucose (Merck) by stomach tube.
	12.10–1.10 p.m.	2.37	2.96	0.80	
	1.20–2.20	3.37	4.28	0.79	200 cc. 0.5 per cent Na_2CO_3 by vein, finished at 3.50 p.m.
	5.04–5.54	2.45	2.87	0.86*	4.00 p.m. 20 gm. glucose (Merck) by stomach tube.

* This was a perfect period, but the apparatus developed a leak in the one following and the experiment had to be discontinued.

The peculiar circumstance in connection with this animal was that the pancreas was found to be sclerotic and brittle. It shelled out of its sheath almost like cold fat. Whether, in view of this fact and the absence of anything resembling pancreatic cells in the surrounding tissues, the case should be classed as one demonstrating the benefit of fasting, we are uncertain. There was

[2] See Paper I of this series.

a positive reaction to the alkali and sugar but the case is atypical among our positive results with totally depancreatized animals. It behaved more like a partially operated dog (see below).

Dog 20, operated 10 days previous to the observations on the respiratory metabolism, had eaten well and had exhibited perfect Minkowski ratios. On Dec. 21, 1914, on a meat and lard diet, the G: N in the morning urine was 2.72. Sodium carbonate was given with the food on this day and the next morning the G: N had fallen to 1.62. On the 23rd, following meat and lard and more carbonate on the evening of the 22nd, the urine drawn by catheter at 9.55 was clear of sugar. The blood sugar had fallen to 0.11 per cent.

TABLE IV.

Dog 20. Weight 9.5 Kg. Pancreatectomy December 11, 1914.

Date.	Time.	Urine.				Respiratory metabolism.			Remarks.
		Source.	Total glucose.	Total nitrogen.	G : N	CO_2	O_2	R. Q.	
1914						*liters*	*liters*		
Dec. 21	9.45 a.m.	Catheter.	4.76	1.75	2.72				
	1.10 p.m.	"	3.33	0.88	3.79				
	6.00 "								300 gm. meat, 30 gm. lard, 15 gm. bone ash, 2 gm. Na_2CO_3.
" 22	10.05 a.m.	"	16.78	10.34	1.62				
	1.10 p.m.	"	0.0	1.19					
	1.15 "								Same diet. All eaten before 4.30. 2 gm. Na_2CO_3 extra at 5 p.m.
" 23	9.45 a.m.	Cage.	2.07	12.00	0.17				
		Catheter.	0.0						
	9.55								0.11 per cent sugar in blood.
	10.00–10.15	30 gm. glucose + 2 gm. Na_2CO_3 by vein.							
	11.30								0.40 "
	12.23– 1.23					2.746	3.051	0.90	
	1.23– 2.23					2.886	3.387	0.85	
	2.23– 3.23					2.625	3.072	0.85	
" 24	Autopsy: No pancreatic tissue.								

When 30 gm. of sugar and 2 gm. of sodium carbonate (anhydrous) were given by vein at 10 a.m. and the dog was placed in the respiration apparatus, quotients of 0.90, 0.85, and 0.85, successively, were obtained. The dog had eaten well up to the night before and seemed in good condition for the experiment but was found dead the next day, and we had no opportunity to establish further controls. It may be remarked here that the same glucose (Merck's dextrose), when aerated with a 1 per cent solution of sodium carbonate, gave off no carbon dioxide. Hence, the result is probably due to a true combustion, but a combustion which cannot of necessity be ascribed to the treatment. Probably every student of pancreatic diabetes in the dog has witnessed improvements, especially as regards the excretion of sugar, a day or two previous to the death of the animal. Minkowski (8) states that sugar, which at the height of the diabetes is excreted, will, at a stage when the animal is scarcely able to move, be held back, and that twice in his long experience he had seen sugar entirely disappear from the urine. Minkowski thought the change was due to a falling off in sugar production rather than the vicarious participation of some other organ.[3] Whatever explanation is finally found for the disappearance of sugar will probably cover the apparent improvement now ascertained, we believe, for the first time,[4] regarding the respiratory metabolism.

C. Positive Effects with Sodium Hydroxide and Pancreatic Extract.

Three instances of clear-cut beneficial effects on the respiratory metabolism of completely depancreatized dogs by the administration of sodium hydroxide in Ringer's solution may now be cited. In two of them pancreatic extract was given with the alkali.

Dog 45 exhibited on the 2nd day after operation an average R. Q. of 0.70 and the same 3 days later. Since the R. Q. seems to be a safer criterion than the G:N ratio, one feels no hesitation in saying that this dog, fed right along on meat diet, was actually an instance of total diabetes. Nevertheless, by administration of 175 cc. of 0.1 N sodium hydroxide in Ringer's solution, the R. Q. was raised some five points and

[3] Minkowski (8), page 110.

[4] Several other instances of this could be cited from our own results.

TABLE V.

Dog 45. Young Female. Weight about 7.0 Kg. Pancreatectomy December 2, 1915.

Date.	Weight.	Temperature.	Time.	Respiratory metabolism.			CO_2 in venous blood.	Sugar in blood.
				CO_2	O_2	R. Q.		
1915	*kg.*	*°C.*		*liters*	*liters*		*per cent*	*per cent*
Dec. 3		38.2						
" 4	6.80	38.2	11.45– 1.03	4.290	5.944	0.72		
			1.03– 1.45	2.252	3.324	0.68		
" 7	6.35	38.0	10.26–11.26	2.593	3.773	0.69		
			11.26–12.26	2.832	3.946	0.72	52.17	0.29
			1.05– 1.20	Infusion by jugular vein of 175 cc. Ringer's solution + 0.1 N NaOH.				
			2.55– 3.55	3.067	4.019	0.76		
			3.55– 5.00	3.322	4.414	0.75	65.52	0.24

the total heat production, as judged by the oxygen intake, was also augmented. The larger percentage of CO_2 in the venous blood shows that the R. Q. would have been even higher if all had been eliminated. The dog was quiet throughout. No sugar was given, but there was abundance of it in the circulation and, if the dynamic effect and the rise in R. Q. together do not indicate combustion, then we have no criteria which can be relied upon.

Dog 51, by all criteria was completely diabetic on the 3rd day following pancreatectomy. Ringer's solution[5] plus 1 per cent sodium carbonate by vein did not affect the R. Q. on the 6th day although the G: N was distinctly lower 2 days later and a second dose did not reduce it further; in fact, there was a slight rise in G: N. 4 days after this—the 12th since operation—a dose of Ringer's solution, in which the pancreas of two dogs had been allowed to macerate under toluene for 2 months in the ice box, was made alkaline to the extent of 0.05 N by addition of pure sodium hydroxide and was given with 30 gm. of pure dextrose by stomach tube. Immediately preceding the experiment the R. Q. had been at the level of complete diabetes.[6] The effect on the R. Q. and on the heat production was seen within an hour. Some doubt was thrown on the results by vomit-

[5] Adding sodium carbonate to Ringer's solution precipitates the calcium as carbonate, but this does not interfere with its administration intravenously, as the carbonate probably rapidly becomes bicarbonate by taking up more carbon dioxide.

[6] Murlin (7). The probable error on the respiratory quotient for two successive periods with the respiration incubator which was used in all this work is not more than ± 0.02.

TABLE VI.

Dog 51. Pancreatectomy May 17, 1916.

Date.	Weight.	Time.	Glucose per hr.	Nitrogen per hr.	G : N.	Respiratory metabolism.			Calories per hr.	Remarks.
						CO_2	O_2	R. Q.		
1916	*kg.*					*liters*	*liters*			
May 20	9.55	9.28 a.m.								Dog fed standard diet of meat and lard.
		9.59–10.59				4.56	6.69	0.68	30.85	
		10.59–11.59				4.45	6.14	0.71	28.30	
		12.10 p.m.	1.00	0.30	3.3					
" 23	9.00	10.00 a.m.								
		10.40–11.40				4.06	5.62	0.72	25.66	
		11.40–12.40				3.69	5.15	0.72	23.37	
		1.52 p.m.	0.66	0.30	2.2					
		2.45–3.00								Given 140 cc. 1 per cent Na_2CO_3 in Ringer's solution by jugular vein.
		3.22– 4.22				4.09	5.97	0.70		Dog urinated in cage and defecated.
" 25		9.35 a.m.			1.8					
		11.35 "	0.79	0.46	1.7					
		12.25–12.45								Injected 150 cc. Ringer's solution freshly made + 1 per cent Na_2CO_3 by femoral vein.
		2.35 p.m.	0.86	0.53	1.6					
		4.35 "	1.17	0.50	2.4					
" 29	7.90	9.53 a.m.			0.63					
		10.49–11.49				2.78	4.20	0.66	19.79	
		11.49–12.49				3.19	4.67	0.68	22.03	
		1.05 p.m.	0.36	0.25	1.5					
		1.25 "								Given 200 cc. Ringer's solution + 0.05 N NaOH + pancreas extract + 30 gm. glucose by stomach.
		2.28– 3.28				4.38	5.88	0.75		Dog vomited in cage.
		3.28– 4.28				4.36	5.58	0.78		"

TABLE VI—*Concluded.*

Date.	Weight.	Time.	Glucose per hr.	Nitrogen per hr.	G : N.	Respiratory metabolism.			Calories per hr.	Remarks.
						CO_2	O_2	R. Q.		
1916	*kg.*					*liters*	*liters*			
May 31	7.55	10.03 a.m.			1.8					
		10.10 "								Given 30 gm. glucose by stomach tube.
		12.14– 1.14				3.58	4.57	0.79	20.39	
		1.14– 2.14				3.57	4.61	0.77	20.39	
		2.32 p.m.		0.52						
		2.36 "								Given 30 gm. glucose in Ringer's solution + 0.05 N NaOH + pancreas extract by stomach tube.
		3.04– 4.04				3.74	4.76	0.79	21.30	
		4.04– 5.04				4.42	5.09	0.87	23.41	
June 1	7.35	9.05 a.m.								
		9.42–10.42				2.77	3.54	0.78		
		10.42–11.42				3.12	4.01	0.78		
		1.52 p.m.	0.20	0.13	1.5					
" 2	7.25	9.46 a.m.								
		10.27–11.27				2.85	3.65	0.78		
		11.27–12.27				3.21	4.42	0.72		
		12.45 p.m.								200 cc. Ringer's solution + 0.05 N NaOH + pancreas extract + 50 gm. glucose by stomach tube.
		1.55– 2.55				3.16	4.11	0.76		
		2.55– 3.55				3.55	4.80	0.72		
		3.55– 4.55				4.46	5.74	0.75		

ing in the respiration chamber and the experiment was repeated 2 days later. To our surprise, the R. Q. after sugar alone was even higher than before, but again the combination of alkali, pancreas extract, and sugar in Ringer's solution carried it to a higher level. This time the heat production could be accurately calculated because the urinary nitrogen was obtainable and showed again what was clearly indicated by the rise in oxygen absorption in the first instance; namely, a considerable dynamic effect from the increased combustion. Subsequent attempts to raise the quotient still further by giving more glucose did not succeed. It is worthy

of note, however, that Dog 51 lived for 11 days following the last attempt and continued to eat a mixed diet for more than a week.

Several animals (*e.g.*, Dog 54) were sacrificed in the attempt to repeat this experiment on Dog 51 but with only partial success thus far. An increase of ten points in the quotient, with an accompanying dynamic action, in an animal known to be completely deprived of internal pancreatic function at a time when the deficiency was most serious, is the most hopeful sign we have encountered in our work that it may yet be possible artificially to restore this function not only to operated dogs but also to men suffering with diabetes mellitus. Consequently, we have spared no pains to repeat the experimental conditions under which the above results were obtained.

Dog 53 is the only other animal which has yielded profitable results. Here again the dog is known to have been completely diabetic at the start. The first administration of alkaline Ringer's solution containing pancreas extract and sugar raised the quotient very distinctly the second hour, and by a second trial the quotient was kept at a level indicating the combustion of protein with all the glucose it can yield. The third attempt (on June 29) resulted in only partial success as there was evidently a leak in the apparatus the first hour. The dog was fed a large amount of carbohydrate following this experiment, with the hope of filling up the glycogen reservoirs and of obtaining something approaching normal combustion the next day, but the next morning the dog was found dead from some unknown cause. The respiration apparatus was checked frequently by combustion of alcohol during this season of work and is known to have been in perfect condition.

Possible Rôle of Pancreatic Extract.

So far as known to the writers, nobody hitherto has prepared a pancreatic extract on the assumption that the alkali which the pancreas is capable of producing may be of some significance for its internal function as well as for its external enzymes. As will be seen from Paper IV of this series,[7] there is some ground for such an assumption; hence, it would seem that a pancreatic extract designed to replace the lost hormone should be contained in an alkaline medium. Allen (9) has emphasized the fact that pancreatic extracts cannot be expected to replace the natural

[7] To be published later.

TABLE VII.

Dog 53. Pancreatectomy June 23, 1916.

Date.	Weight.	Time.	Respiratory metabolism.			Remarks.
			CO_2	O_2	R.Q.	
1916	*kg.*		*liters*	*liters*		
June 26	6.33	11.10–12.10	3.01	4.48	0.67	250 gm. lean beef for diet.
		12.10– 2.05	3.01	4.40	0.69	
		2.05– 3.05	2.44	3.33	0.73	
" 27	6.25	10.04–11.00	3.13	4.22	0.70	
		11.00–12.00	3.33	4.74	0.70	
		12.05				Given 200 cc. Ringer's solution + pancreas extract + 0.05 N NaOH + 30 gm. dextrose by stomach tube.
		1.14– 2.14	3.72	5.16	0.72	
		2.14– 3.14	3.70	4.45	0.83	
" 28		12.30				Given 200 cc. Ringer's solution + pancreas extract + 0.05 N NaOH + 30 gm. dextrose by stomach tube.
	6.40	1.07– 2.07	3.92	5.09	0.77	
		2.07– 3.07	3.72	4.69	0.79	
		3.07– 4.07	4.10	5.68	0.72	
" 29	5.90	10.00–11.00	3.05	4.11	0.74	
		11.00–12.00	2.76	3.59	0.77	
		12.45				Given 200 cc. Ringer's solution + pancreas extract + 0.05 N NaOH by stomach tube.
		3.35– 4.35	2.92	4.30	0.68?	Leak.
		4.35– 5.35	2.60	3.30	0.79	

product, which is essential to the combustion of carbohydrate, unless the pancreatic cells are preserved. Acting on this suggestion and emboldened by the announcement of Kleiner and Meltzer (10) that an "emulsion" of pancreatic material had appeared to afford some assistance in disposing of extra sugar injected into the circulation of diabetic animals, we prepared the extracts used in the preceding experiments by grinding fresh dog pancreas in Ringer's solution and placing the resulting suspen-

TABLE VIII.

Dog 37. Partial Pancreatectomy August 3, 1915, Leaving About 2 Gm.

Date.	Weight.	Nature of experiment.	Respiratory period.	Respiratory metabolism.			Calories per hr.	Remarks.
				CO_2	O_2	R. Q.		
1915	*kg.*			*liters*	*liters*			
Aug. 9	6.0	Basal.	2.34– 3.34	2.328	3.220	0.72	15.14	Fasting 24 hrs.
			3.34– 4.38	2.385	3.154	0.76	14.99	
" 11	5.7	"	1.02– 2.02	2.235	3.073	0.73	14.49	Standard diet Aug. 10 5 p.m.
			2.02– 3.02	2.317	2.682	0.86	13.08	
			3.02– 4.02	2.300	3.023	0.76	14.36	
" 20	5.7	"	3.22– 4.22	2.457	3.247	0.76	15.43	Standard diet Aug. 19 5 p.m.
			4.22– 5.22	2.344	3.016	0.78	14.41	
			Average	2.338	3.059	0.77	14.55	
" 23	5.55	After 50 gm. glucose at 3.45.	5.05– 6.05	2.463	2.920	0.84	14.16	
			6.05– 7.05	2.392	2.675	0.89	13.14	
			7.05– 8.05	3.003	3.281	0.92	16.24	Fasting previous 24 hrs.
" 27	6.1	After 50 gm. glucose at 10.15 a.m.	10.52–11.52	2.520	3.001	0.84	14.56	Standard diet 3 days.
			11.52–12.52	2.616	2.913	0.90	14.34	
			12.52– 1.52	2.813	3.210	0.88	15.72	
			Average	2.635	3.000	0.88	14.69	
" 28	6.1	After 50 gm. glucose and 3 gm. Na_2CO_3 at 9.23.	11.13–12.13	2.963	3.089	0.96	15.44	
			12.13– 1.13	3.581	4.160	0.86		Dog vomited at 12.22.
			1.13– 2.13	2.968	2.937	1.01	14.83	

Aug. 31	5.85	Basal.	10–57–11.57	2.293	3.151	0.73	14.85	Standard diet Aug. 30
			11.57–12.57	1.997	2.480	0.80	12.81	5 p.m.
			12.57– 1.57	2.361	3.168	0.74	15.02	
			Average	2.217	2.933	0.76	14.23	
" 31	5.85	After 3 gm. Na_2CO_3 alone at 2.45.	3.56– 4.56	1.921	2.568	0.75	12.17	
			4.56– 5.56	2.191	2.835	0.77	13.51	
			Average	2.056	2.701	0.76	12.84	
Sept. 4	6.4	50 gm. glucose + 3 gm. Na_2CO_3 at 9.45.	10.35–11.35	2.506	3.051	0.82	14.73	Standard diet Sept. 3
			11.35–12.35	2.739	2.820	0.97	14.13	5 p.m.
			12.35– 1.35	3.004	3.380	0.89	16.63	
			1.35– 2.35	2.939	3.275	0.90	16.13	
			Average	2.797	3.131	0.90	15.40	
" 6	6.3	50 gm. glucose at 1.35.	3.17– 4.17	2.178	2.825	0.77	13.45	Standard diet Sept. 5
			4.17– 5.17	2.372	3.003	0.79	14.40	5 p.m.
			5.17– 6.20	2.493	3.228	0.77	14.64	
			Average			0.78	14.18	
" 8	6.0	After 3 gm. Na_2CO_3 at 9.40.	10.36–11.36	2.087	2.620	0.80	12.58	Standard diet Sept. 7
			11.36–12.37	2.078	2.812	0.74	13.30	5 p.m.
			12.37– 1.37	2.031	2.460	0.83	11.91	
			Average	2.085	2.631	0.79	12.59	
" 9	6.2	Basal.	10.06–11.06	2.341	3.505	0.67	16.43	Standard diet Sept. 8
			11.06–12.06	2.022	2.149	0.94	10.65	5 p.m.
			12.06– 1.06	2.658	3.418	0.78	16.28	
			Average	2.340	3.024	0.79	14.44	

sion in the ice box for several weeks. At the end of this time it was filtered through cloth rendered alkaline, as already described, and administered. The two successes recorded are possibly confirmatory of the findings of Kleiner and Meltzer although it is impossible to state this positively until the authors reveal their method of preparing their pancreatic "emulsion." It is also possible that the failures which we are obliged to admit may be due to some variation in the mode of preparation of the pancreatic material, to the length of time the material was permitted to extract in the ice box, for example, or to the interval which elapsed between the time the pancreas was removed and treatment begun.

In view of the fact that we have had many failures to influence the respiratory quotient more than two or three points with alkali and glucose alone (Table V, page 491) on animals anatomically completely deprived of the pancreas, and in view of the much more striking effects to be seen presently on partially operated animals, our inclination at present is to look upon Dog 45, treated with alkali alone, as one which had some residual potential for oxidation of glucose, and that the alkali only restored a missing condition.

Partially Depancreatized Dogs.

Dog 37 was deprived of its pancreas on Aug. 3, 1915, only about 2 gm. of pancreatic tissue being left around the duct. The method of procedure, in brief, was as follows: The basal metabolism was determined for two or more hourly periods on different days and also preliminary to the administration of glucose or carbonate or both. Having determined the basal metabolism, 50 gm. of glucose were given by stomach tube (if not promptly taken by the dog when offered with the food) and the respiratory exchange determined for several hourly periods. On subsequent days 50 gm. of glucose and 3 gm. of sodium carbonate or 3 gm. of carbonate alone were given *per os* and the previous procedure repeated. The results are given in Table VIII.

It will be noted that from Aug. 9 to Sept. 9 the basal metabolism as measured by the oxygen consumption remained practically the same; likewise the basal respiratory quotient. Nevertheless, while on Aug. 23 the administration of 50 gm. of glucose was followed by an average rise in quotient of eleven points, the

administration of an equal quantity of glucose on Sept. 6 produced an increase in quotient of but two points and that in only one period. The administration of carbonate along with the glucose raised the respiratory quotient on Aug. 28 to an average of 0.94, or seventeen points above the basal level, and on Sept. 4 to 0.90, or thirteen points above the basal level, although the administration of carbonate alone, both on Aug. 31 and Sept. 8, produced no effect on the respiratory quotient. It is worthy of note that the total heat production after carbonate was lower than the basal metabolism. To sum up the observations on this dog, we may say that a partially depancreatized dog fed on "standard diet" (consisting of 150 gm. of lean meat, 30 gm. of lard, and 50 gm. of cracker meal) with the occasional addition of 50 gm. of glucose showed a gradual loss in ability to oxidize glucose, and that this function was decidedly improved in each instance by the oral administration of sodium carbonate with the glucose. The fact that the administration of carbonate alone was followed neither by a rise in quotient nor by an increase in carbon dioxide elimination per hour makes us believe that the rise in quotient after carbonate and sugar can mean only increased glucose oxidation.

TABLE IX.

Dog 42. Control of Effect of Na_2CO_3 on the Normal Dog.

Date.	Food, etc.	Period.	Respiratory metabolism.		
			CO_2	O_2	R. Q.
1915			*liters*	*liters*	
Sept. 22	Standard diet* previous day.	1	3.787	5.103	0.74
		2	2.912	3.878	0.75
" 23	Standard diet previous day 5 p.m.	1	3.032	3.941	0.77
		2	3.188	4.271	0.75
		3	3.317	4.477	0.74
" 24	Standard diet and 6 gm. Na_2CO_3 evening before. 200 cc. 1 per cent Na_2CO_3 just before experiment.	1	4.066	5.411	0.75
		2	3.761	4.820	0.78
		3	2.869	3.445	0.79†

* Beef heart, lard, and cracker meal. † Note lower CO_2.

In spite of these carefully controlled observations, it might still be contended, as intimated in the first part of this paper, that the rise in quotient after administration of combined glucose and carbonate was due to liberation of carbon dioxide from the salt by the hydrochloric acid of the gastric juice or that the carbonate might itself give off carbon dioxide while circulating in the blood. To satisfy ourselves that such was not the case, two experiments were done on normal dogs, Nos. 42 and 43. In one of these experiments the basal metabolism was determined after 24 and again after 72 hours of fasting, and then 2.5 to 5 gm. of sodium carbonate were given by mouth and the respiratory exchange determined for several hourly periods. As seen in the

TABLE X.

Dog 43. Control of Effect of Na_2CO_3 on Normal Dog.

Date.	Previous diet.	Experiment.	Period.	Respiratory metabolism.			N per hr.	Calories per hr.
				CO_2	O_2	R. Q.		
1915				*liters*	*liters*			
Sept. 28	Fasting 24 hrs.	Basal.	1	3.056	4.185	0.73	0.166	19.42
			2	3.076	4.284	0.72	0.166	19.88
			3	2.975	3.928	0.76	0.166	18.30
			Average	3.035	4.132	0.74		19.20
" 29	Fasting 48 hrs. after 5 gm. Na_2CO_3 the evening before and 2 gm. Na_2CO_3 in 300 cc. water just before the experiment.							
			1	3.294	4.502	0.73	0.194	20.90
			2	3.209	4.017	0.80	0.194	18.93
			3	3.142	4.460	0.70	0.194	20.67
			Average	3.215	4.326	0.74		20.17
" 30	Fasting 72 hrs.		1	3.434	4.424	0.78	0.142	20.86
			2	3.522	5.010	0.70	0.142	23.32
			3	3.088	3.818	0.81	0.142	18.11
			Average	3.348	4.417	0.76		21.09
Oct. 1	Fasting.		1	2.976	3.922	0.76		
			2	3.000	3.919	0.77		
" 4	"		1	2.718	3.580	0.78		
			2	2.538	3.409	0.74		
	2.5 gm. Na CO_3 in 400 cc. water.							
			1 and 2	6.246	8.102	0.77		
			3	3.561	4.655	0.76		

table, no appreciable effect, either on the amount of carbon dioxide eliminated, or on the respiratory quotient, was obtained. An experiment on Dog 42 that received the standard diet gave equally negative results.

Dog 57 was depancreatized on Aug. 21, 1916. A fragment weighing about 1.5 gm. was left around the main duct. 200 gm. of lean meat were given daily. On Sept. 2 a study of the basal metabolism gave quotients of 0.67 and 0.68 in successive periods, indicating that ability to oxidize glucose was completely lost. This was confirmed by the fact that after the administration of 50 gm. of glucose the respiratory quotient rose but two points. The next morning 200 cc. of 1 per cent sodium carbonate solution was administered intravenously and the respiratory exchange studied for two periods. The respiratory quotient rose to 0.72 during the first period but during the second period dropped to 0.59, which was found to be due to a leak in a valve. After the administration of 50 gm. of glucose and about 5½ hours after the injection of the alkali, the respiratory quotient rose to 0.88 in the first, and 0.96 in the third period, both of which periods were obtained with the same set of absorbers. The low quotient of 0.65 was undoubtedly due to a leak.

TABLE XI.

Dog 57. Female. Weight 13 Kg. Partial Pancreatectomy, About 1.5 Gm. of Pancreas Left around the Main Duct.

Date.	Time.	Weight.	Respiratory metabolism.			Remarks.
			CO_2	O_2	R. Q.	
1915		*kg.*	*liters*	*liters*		
Sept. 2	4.09– 4.54	11.35	3.24	4.93	0.67	Basal.
	4.54– 5.58		4.94	7.10	0.68	
	6.05					50 gm. glucose *per os*.
	8.15– 8.55		2.97	4.22	0.70	
" 3	11.15–11.45					200 cc. of 1 per cent Na_2CO_3 intravenously.
	1.16– 2.02	10.75	4.62	6.39	0.72	
	2.03– 2.32		1.79	3.12	0.59	Valve leaked.
	4.15					50 gm. glucose *per os*.
	4.32– 5.16		2.75	3.13	0.88	
	5.16– 5.59		2.995	4.61	0.65	Valve leaked.
	5.59– 6.44		4.04	4.10	0.96	

Thus, the administration of glucose or carbonate alone caused but a slight rise in the respiratory quotient, while the administration of glucose at a time when the urine would otherwise have

been sugar-free and when the carbonate must still have been circulating in the blood, as shown in previous experiments, in the first paper of this series, yielded a very significant increase in the respiratory quotient. This dog, anatomically speaking, still had some pancreas, but functionally, as indicated by the respiratory quotient, had completely lost the ability to oxidize sugar. It is worthy of note here that the observations on Dog 37 are confirmed by this experiment in which the alkali was injected directly into the blood and hence could not have come in contact with the hydrochloric acid of the gastric juice; also that Dog 57 received 200 gm. of lean beef daily. It is, we believe, a significant fact that both these partially depancreatized dogs seem to have lost their power to burn glucose to a large extent, while subsisting on a diet containing meat.

DISCUSSION.

We shall not attempt at this time a full explanation of the facts presented in this series of papers; but it will be advantageous to focus attention upon the most important facts that have been established beyond a reasonable doubt.

1. In completely depancreatized dogs the administration of sodium carbonate, whether by mouth or by the intravenous route, is promptly followed by a retention of considerable amounts of glucose of endogenous or exogenous origin. The retention is rarely, if ever, accompanied by any evidence of improved oxidation of glucose.

2. In partially depancreatized dogs which still have some capacity to oxidize glucose, the administration of sodium carbonate or sodium hydroxide with glucose is followed by a greater oxidation of glucose than when either is given alone.

3. Sodium carbonate itself causes but a slight increase in respiratory quotient, or none, whether administered *per os* or intravenously, to normal or diabetic dogs.

Thus, it seems that while sodium carbonate or sodium hydroxide alone cannot restore the lost function to an organism completely deprived of its ability to oxidize glucose, either substance can improve the capacity of the organism as a whole to oxidize glucose when this function is more or less crippled although not completely lost.

The importance of alkalies, particularly sodium carbonate and the alkaline sodium phosphates, in glycolysis (11) is well known. Rona and Döblin (12), as well as Rona and Arnheim (13), have called attention to the fact that the addition of phosphates or carbonates to blood increases its glycolytic power *in vitro*. More recently, Levene and Meyer (14) were able to demonstrate a conversion of glucose into lactic acid by leukocytes in the presence of an alkaline phosphate mixture under aseptic conditions. Thus, the ability of alkalies, particularly sodium carbonate and the alkaline sodium phosphates, to facilitate a primary cleavage of glucose by at least one body tissue, the blood, seems conclusively established. The manner in which this is brought about is as yet imperfectly understood.

Warburg (15) has shown that sodium hydroxide greatly increases the oxidation of fertilized eggs of the sea urchin although it does not enter the egg. Osterhout (16) concludes that "small amounts of NaOH are able to produce a considerable increase of permeability" and calls attention to the fact that "increasing the permeability of the egg for products of oxidation would increase oxidation." Loeb and Wasteneys (17) confirmed the observations of Warburg, but found that with *Arbacia* eggs ammonium hydroxide also increased the rate of oxidation. "The fact that NaOH acts more powerfully than NH_4OH" in the same concentration of substance, they believe, "excludes the idea that we are dealing here with an effect of the undissociated molecule, and the parallelism in the effects of the two bases suggests a common source of their action;" namely, the OH ion. "The fact that the weak base acts more powerfully than we should expect from its degree of dissociation," may be ascribed, they think, to its greater power of penetration into the egg. "In this case one can explain all the phenomena on the assumption that while the greater part of oxygen is consumed at the surface of the egg, some diffuses into the egg and is consumed in the layers adjacent to the surface."

In view of the lack of definite knowledge as to the conditions under which the cleavage of the glucose molecule is brought about or the manner in which this process takes place, any attempt to describe the mechanism whereby the oxidation of glucose in the depancreatized dog is accelerated must, for the

present, be mere speculation. It might be suggested that sodium hydroxide or sodium carbonate accelerates the oxidation of sugar, by increasing the permeability of the cell for oxygen or glucose or for both, or that the union of the alkaline salt with the sugar forms a combination that can be fixed at the surface of the cell and there oxidized. On the other hand, the alkali may act by creating a more favorable environment for the glycolytic action of a cell which still possesses the potential capacity to burn glucose.

BIBLIOGRAPHY.

1. Lusk, G., *J. Biol. Chem.*, 1915, xx, 555.
2. Lusk, *Arch. Int. Med.*, 1915, xv, 939.
3. La Franca, S., *Z. exp. Path. u. Ther.*, 1909, vi, 1.
4. Falta, W., Grote, F., and Staehelin, R., *Beitr. chem. Phys. u. Path.*, 1907, x, 199.
5. Murlin, J. R., and Kramer, B., *J. Biol. Chem.*, 1913, xv, 365.
6. Murlin, J. R., *Am. J. Physiol.*, 1910, xxvi, 134.
7. Murlin, *Am. J. Dis. Child.*, 1915, ix, 43.
8. Minkowski, O., *Arch. exp. Path. u. Pharm.*, 1893, xxxi, 106.
9. Allen, F. M., Glycosuria and Diabetes, Boston, 1913, 128.
10. Kleiner, I. S., and Meltzer, S. J., *Proc. Nat. Acad. Sc.*, 1915, i, 338.
11. Landsberg, M., *Deutsch. Arch. klin. Med.*, 1914, cxv, 465.
12. Rona, P., and Döblin, A., *Biochem. Z.*, 1911, xxxii, 489.
13. Rona, P., and Arnheim, F., *Biochem. Z.*, 1912–13, xlviii, 35.
14. Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1912, xi, 347, 353, 361; 1912, xii, 265.
15. Warburg, O., *Z. physiol. Chem.*, 1910, lxvi, 305; *Biochem. Z.*, 1910, xxix, 414.
16. Osterhout, W. J. V., *J. Biol. Chem.*, 1914, xix, 335.
17. Loeb, J., and Wasteneys, H., *J. Biol. Chem.*, 1915, xxi, 153; see also *ibid.*, 1913, xiv, 355.

INDEX TO VOLUME XXVII.

ADDIS, T., and WATANABE, C. K. The rate of urea excretion. II. The rate of excretion of administered urea in young healthy adults on a constant diet, 249

——, and ——. The volume of urine in young healthy adults on a constant diet, 267

Acetone in urine, colorimetric method for determination of, 209

Acid and alkali, influence on glycosuria and hyperglycemia, 481

——, isobutyric, action of hydrogen peroxide on homologues of, 433

Acidity of plant cells shown by natural indicators, 233

Acidosis and carbohydrate metabolism, relationship of creatinuria to, 151

—— and creatine elimination, possible interrelations between, 127

Acids and alkalies, permeability to, of living cells, 225

——, amino-, of blood serum, influence of ether anesthesia on, 45

——, amino-, and carbohydrates, reaction between, as probable cause of humin formation, 71

——, fatty, branched chain, oxidation of, 433

—— and potassium salts, analogy for, of mechanism of diffusion (of electrolytes through membranes of living cells), 363

Alkali and acid, influence on glycosuria and hyperglycemia, 481

—— blue in organism, 377

Alkali, influence on creatine elimination during inanition, 141

——, influence on creatinuria of phlorhizin glycosuria, 147

——, influence on respiratory metabolism after total and partial pancreatectomy, 517

Alkalies and acids, permeability to, of living cells, 225

ALLEN, C. H. See LEVENE and ALLEN, 433

Amino-acids of blood serum, influence of ether anesthesia on, 45

—— and carbohydrates, reaction between, as probable cause of humin formation, 71

Ammonia and urea in muscle, direct determination of, 95

Anesthesia, ether, influence on amino-acids of blood serum, 45

Autolysis, influence of bile on, 243

BAUMANN, EMIL J. See UNDERHILL and BAUMANN, 147, 151, 169.

BENGIS, ROBERT. See SALANT and BENGIS, 403

Bile, influence of, on autolysis, 243

Blood fat and blood sugar content of dogs under influence of hydrazine, interrelations of, 169

—— of goats, changes in cholesterol content of, following cholesterol feeding alone, Roentgen treatment alone, and cholesterol feeding combined with Roentgen treatment and subsequent castration, 273

Blood serum, influence of ether anesthesia on amino-acids of, 45
——, small amounts of, separate determination of cholesterol and cholesterol esters in, 107
—— sugar and blood fat content of dogs under influence of hydrazine, interrelations of, 169
BLOOR, W. R., and KNUDSON, ARTHUR. The separate determination of cholesterol and cholesterol esters in small amounts of blood, 107
BODANSKY, A. The chymase of *Solanum elaeagnifolium*, 103
BOGERT, L. JEAN. See UNDERHILL and BOGERT, 161
Bread, white, yeast, and polished rice, nutritive value of, determined by experiments on man, 173
Butter fat and lard in growth, comparative value of, 51

CALORIMETRY, clinical, 217
Carbohydrate metabolism and acidosis, relationship of creatinuria to, 151
Carbohydrates and amino-acids, reaction between, as probable cause of humin formation, 71
Carbonate of sodium administered to depancreatized dogs, is glucose retained deposited as glycogen, after, 499
Carotin, toxicity of, 213
Carotinoids of plant, and of cow, horse, sheep, goat, pig, and hen, physiological relation of, 27
Castration following cholesterol feeding combined with Roentgen treatment, changes in cholesterol content of blood of goats after, 273
Cells, living, mechanism of diffusion of electrolytes through membranes of, 339, 353, 363
Cells, living, permeability of, to acids and alkalies, 225
—— of plant, acidity of, shown by natural indicators, 233
Chloride, potassium, diffusion of, out of egg of *Fundulus*, and relative efficiency of different ions for salt effect, 353
Cholesterol absorption, mechanism of, 463
—— added to diet deficient in fats, influence on growth of white mouse, 393
—— and cholesterol esters, separate determination of, in small amounts of blood, 107
—— content of blood of goats, changes in, following cholesterol feeding alone, Roentgen treatment alone, and cholesterol feeding combined with Roentgen treatment and subsequent castration, 273
Chymase of *Solanum elaeagnifolium*, 103
Coal tar colors, physiological and pharmacological studies on, 403
Colorimetric method for determination of acetone in urine, 209
Colors, coal tar, physiological and pharmacological studies on, 403
Corn, nutritive properties of, 193
Cottonseed meal as an incomplete food, 15
Creatine elimination and acidosis, possible interrelations between, 127
—— elimination during inanition, influence of alkali upon, 141
—— metabolism, studies in, 127, 141, 147, 151
Creatinuria of phlorhizin glycosuria, influence of alkali upon, 147
——, relationship of, to carbohydrate metabolism and acidosis, 151

Csonka, Frank A. A colorimetric method for the determination of acetone in urine, 209

Desiccator, vacuum, electrically heated, 429
Determination of acetone in urine, colorimetric method for, 209
——, direct, of urea and ammonia in muscle, 95
—— of nitric nitrogen, method for, 327
——, separate, of cholesterol and cholesterol esters in small amounts of blood, 107
Diabetes, pancreatic, in the dog, 481, 499, 517
Diet, changes in, determining alterations in output of urinary constituents, 161
——, constant, urine volume in young healthy adults on, 267
—— deficient in fats and same diet with cholesterol added, influence on growth of white mouse, 393
——, rate of excretion of administered urea in young healthy adults on constant diet, 249
——, relation of unidentified factors (fat-soluble *A* and water-soluble *B*) of, to growth-promoting properties of milk, 33
Dietary conditions bearing on problem of growth in rats, 1
Du Bois, Eugene F. See Gephart, Du Bois, and Lusk, 217
Dyes, fat-soluble, experiments with, 403

Eckles, C. H., and Palmer, Leroy S. The influence of parturition on the composition and properties of the milk and milk fat of the cow, 313
Eddy, Walter H. The isolation of a growth-producing substance from sheep pancreas, 113
Egg of *Fundulus*, diffusion of KCl out of, and relative efficiency of different ions for salt effect, 353
Elaeagnifolium, *Solanum*, chymase of, 103
Electrolytes, mechanism of diffusion of, through membranes of living cells, 339, 353, 363
Esters of cholesterol, and cholesterol, separate determination of, in small amounts of blood, 107
Ether anesthesia, influence on amino-acids of blood serum, 45
Evolution and nutrition, 309
Ewing, P. V. See Wells and Ewing, 15

Fat of blood and blood sugar content of dogs under influence of hydrazine, interrelations of, 169
—— of butter, and lard, in growth, comparative value of, 51
—— of cow's milk, influence of parturition on composition and properties of, 313
Fat-soluble *A* and water-soluble *B*, unidentified factors of diet, relation of, to growth-promoting properties of milk, 33
—— dyes, experiments with, 403
Fats, diet deficient in, and with cholesterol added, influence on growth of white mouse, 393
Fatty acids, branched chain, oxidation of, 433
Fenger, Frederic. Phosphatides in the ductless glands, 303
Food, incomplete, cottonseed meal as, 15
Fundulus, egg of, diffusion of KCl out of, and relative efficiency of different ions for salt effect, 353
Funk, Casimir. The study of certain dietary conditions bearing on the problem of growth in rats, 1

FUNK, CASIMIR, LYLE, W. G., and MCCASKEY, DONALD. The nutritive value of yeast, polished rice, and white bread, as determined by experiments on man, 173

——, and MACALLUM, ARCHIBALD BRUCE. Studies on growth. III. The comparative value of lard and butter fat in growth, 51. IV. The action of yeast fractions on the growth of rats, 63

GEPHART, FRANK C., DU BOIS, EUGENE F., and LUSK, GRAHAM. Clinical calorimetry. XVIII. The number of places of significant figures in the data of metabolism experiments, 217

Glands, ductless, phosphatides in, 303

Glucose retained when sodium carbonate is administered to depancreatized dogs, deposited as glycogen, 499

Glycogen, glucose deposited as, after sodium carbonate is administered to depancreatized dogs, 499

Glycosuria and hyperglycemia, influence of alkali and acid upon, 481

——, phlorhizin, influence of alkali upon creatinuria of, 147

Growth, comparative value of lard and butter fat in, 51

——, experimental studies on, 393

Growth-producing substance, isolation of, from sheep pancreas, 113

Growth-promoting properties of milk, relation of, to unidentified factors of diet, fat-soluble *A* and water-soluble *B*, 33

Growth of rats, action of yeast fractions on, 63

——in rats, dietary conditions bearing on the problem of, 1

Growth, studies on, 51, 63

—— of white mouse on diet deficient in fats and same diet with cholesterol added, 393

HAAS, A. R. The acidity of plant cells as shown by natural indicators, 233

——. The permeability of living cells to acids and alkalies, 225

HEDENBURG, O. F. See WELLS and HEDENBURG, 213

HOGAN, ALBERT G. The nutritive properties of corn, 193

Humin formation, reaction between amino-acids and carbohydrates as probable cause of, 71

Hydrazine, interrelations of blood fat and blood sugar content of dogs under influence of, 169

Hydrogen ion concentration, relation of changes in, to oxidase reactions, 299

—— peroxide, action on homologues of isobutyric acid, 433

ION concentration, hydrogen, relation of changes in, to oxidase reactions, 299

Ions, relative efficiency of, for salt effect, and diffusion of KCl out of egg of *Fundulus*, 353

Isobutyric acid, action of hydrogen peroxide on homologues of, 433

KNUDSON, ARTHUR. See BLOOR and KNUDSON, 107

KRAMER, B., MARKER, J., and MURLIN, J. R. Pancreatic diabetes in the dog. II. Is the glucose retained when sodium carbonate is administered to depancreatized dogs deposited as glycogen, 499

——. See MURLIN and KRAMER, 481, 517

KURIYAMA, SHIGENOBU. The fate of alkali blue in the organism, 377

LARD and butter fat in growth, comparative value of, 51

LEVENE, P. A., and ALLEN, C. H. The oxidation of branched chain fatty acids. I. The action of hydrogen peroxide on the homologues of isobutyric acid, 433

LOEB, JACQUES. The mechanism of the diffusion of electrolytes through the membranes of living cells. I. The necessity of a general salt effect upon the membrane as a prerequisite for this diffusion, 339. II. The diffusion of KCl out of the egg of *Fundulus* and the relative efficiency of different ions for the salt effect, 353. III. The analogy of the mechanism of the diffusion for acids and potassium salts, 363

——, and NORTHROP, J. H. Nutrition and evolution, 309.

LUDEN, GEORGINE. Observations on the changes in the cholesterol content of the blood of goats, following cholesterol feeding alone, Roentgen treatment alone, and cholesterol feeding combined with Roentgen treatment and subsequent castration, 273

LUSK, GRAHAM. See GEPHART, DU BOIS, and LUSK, 217

LYLE, W. G. See FUNK, LYLE, and MCCASKEY, 173

MACALLUM, ARCHIBALD BRUCE. See [FUNK and MACALLUM, 51, 63

MARKER, J. See KRAMER, MARKER, and MURLIN, 499

MCCASKEY, DONALD. See FUNK, LYLE, and MCCASKEY, 173

MCCOLLUM, E. V., SIMMONDS, N., and PITZ, W. The relation of the unidentified dietary factors, the fat-soluble *A*, and water-soluble *B*, of the diet to the growth-promoting properties of milk, 33

Membranes of living cells, mechanism of diffusion of electrolytes through, 339, 353, 363

Metabolism, carbohydrate, and acidosis, relationship of creatinuria to, 151

—— of creatine, studies in, 127, 141, 147, 151

—— experiments, number of places of significant figures in, 217

——, respiratory, influence of alkali on, after total and partial pancreatectomy, 517

Milk and milk fat of cow, influence of parturition on composition and properties of, 313

——, relation of growth-promoting properties of, to unidentified factors of diet, fat-soluble *A* and water-soluble *B*, 33

MUELLER, J. HOWARD. The mechanism of cholesterol absorption, 463

MURLIN, J. R., and KRAMER, B Pancreatic diabetes in the dog. I. The influence of alkali and acid upon glycosuria and hyperglycemia, 481 III. The influence of alkali on the respiratory metabolism after total and partial pancreatectomy, 517

——. See KRAMER, MARKER, and MURLIN, 499

Muscle, direct determination of urea and ammonia in, 95

NITROGEN, nitric, method for determination of, 327
NORTHROP, J. H. See LOEB and NORTHROP, 309
Nutrition and evolution, 309
Nutritive properties of corn, 193

OXIDASE reactions, relation to changes in hydrogen ion concentration, 299
Oxidation of branched chain fatty acids, 433

PALMER, LEROY S. The physiological relation of plant carotinoids to the carotinoids of the cow, horse, sheep, goat, pig, and hen, 27
——. See ECKLES and PALMER, 313
Pancreas of sheep, isolation of growth-producing substance from, 113
Pancreatectomy, total and partial, influence of alkali on respiratory metabolism after, 517
Pancreatic diabetes in the dog, 481, 499, 517
Parturition, influence on composition and properties of milk and milk fat of cow, 313
Permeability of living cells to acids and alkalies, 225
Peroxide of hydrogen, action on homologues of isobutyric acid, 433
Pharmacological and physiological studies on coal tar colors, 403
Phlorhizin glycosuria, creatinuria of, influence of alkali upon, 147
Phosphatides in the ductless glands, 303
Physiological and pharmacological studies on coal tar colors, 403
—— relation of plant carotinoids to carotinoids of cow, horse, sheep, goat, pig, and hen, 27
PITZ, W. See MCCOLLUM, SIMMONDS, and PITZ, 33
Plant cells, acidity of, shown by natural indicators, 233
Potassium chloride, diffusion of, out of egg of *Fundulus*, and relative efficiency of different ions for salt effect, 353
—— salts, and acids, analogy for, of mechanism of diffusion (of electrolytes through membranes of living cells), 363

REED, GUILFORD B. The relation of oxidase reactions to changes in hydrogen ion concentration, 299
Rice, polished, yeast, and white bread, nutritive value of, determined by experiments on man, 173
ROBERTSON, T. BRAILSFORD. Experimental studies on growth. VIII. The influence of a diet deficient in fats, and of the same diet with cholesterol added, upon the growth of the white mouse, 393
——, and SCHMIDT, CARL L. A. An electrically heated vacuum desiccator, 429
Roentgen treatment alone and with cholesterol feeding, and subsequent castration, changes in cholesterol content of goat's blood after, 273
ROSS, ELLISON L. The influence of ether anesthesia in amino-acids of blood serum, 45
ROXAS, M. L. The reaction between amino-acids and carbohydrates as a probable cause of humin formation, 71

SALANT, WILLIAM, and BENGIS, ROBERT. Physiological and pharmacological studies on coal tar colors. I. Experiments with fat-soluble dyes, 403

Salt effect upon membrane of living cells, necessity of, for diffusion of electrolytes, 339
—— effect, relative efficiency of different ions for, and diffusion of KCl out of egg of *Fundulus*, 353
Salts of potassium, and acids, analogy for, of mechanism of diffusion (of electrolytes through membranes of living cells), 363
SCALES, F. M. A method for the determination of nitric nitrogen, 327
SCHMIDT, CARL L. A. See ROBERTSON and SCHMIDT, 429
Serum of blood, influence of ether anesthesia on amino-acids of, 45
SIMMONDS, N. See MCCOLLUM, SIMMONDS, and PITZ, 33
Sodium carbonate administered to depancreatized dogs, is glucose retained deposited as glycogen, after, 499
Solanum elaeagnifolium, chymase of, 103
Sugar of blood and blood fat content of dogs under influence of hydrazine, interrelations of, 169
SUMNER, JAMES B. The direct determination of urea and ammonia in muscle, 95

TAR (coal) colors, physiological and pharmacological studies on, 403
TATUM, ARTHUR L. The influence of bile on autolysis, 243

UNDERHILL, FRANK P. Studies in creatine metabolism. I. Possible interrelations between acidosis and creatine elimination, 127. II. The influence of alkali upon creatine elimination during inanition, 141.
——, and BAUMANN, EMIL J. Studies in creatine metabolism. III. The influence of alkali upon the creatinuria of phlorhizin glycosuria, 147. IV. The relationship of creatinuria to carbohydrate metabolism and acidosis, 151
——,and—. The interrelations of blood fat and blood sugar content of dogs under the influence of hydrazine, 169
——, and BOGERT, L. JEAN. Alterations in the output of certain urinary constituents as determined by changes in the character of the diet, 161
Urea, administered, rate of excretion of, in young healthy adults on constant diet, 249
—— and ammonia in muscle, direct determination of, 95
—— excretion, rate of, 249
Urinary constituents, alterations in output of, determined by changes in character of diet, 161
Urine, acetone in, colorimetric method for determination of, 209
—— volume in young healthy adults on constant diet, 267

WATANABE, C. K. See ADDIS and WATANABE, 249, 267
Water-soluble *B* and fat-soluble *A*, unidentified factors of diet, relation of, to growth-promoting properties of milk, 33
WELLS, C. A., and EWING, P. V. Cottonseed meal as an incomplete food, 15
WELLS, H. GIDEON, and HEDENBURG, O. F. The toxicity of carotin, 213

YEAST fractions, action of, on growth of rats, 63
——, polished rice, and white bread, nutritive value of, determined by experiments on man, 173